TELOMERE, DNA METHYLATION AND GENE EXPRESSION CHANGES CAUSED BY EXERCISE TRAINING

By

Joshua Denham B.S, M.A

A Thesis submitted in total fulfilment for the requirements of the Doctor of Philosophy (PhD) degree.

Faculty of Science and Technology

Federation University Australia

Federation University Australia
PO Box 663
University Drive, Mount Helen
Ballarat, Victoria 3353,
Australia

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'Statement of authorship'

Except where explicit reference is made in the text of the thesis, this thesis contains no material published elsewhere or extracted in whole or in part from a thesis by which I have qualified for or been awarded another degree or diploma. No other person's work has been relied upon or used without due acknowledgment in the main text and bibliography of the thesis.

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Publications arising from my PhD candidature and my contribution

Paper 1 **Denham, J**., O'Brien, B.J., & Charchar, F.J. (2016). Telomere length maintenance and cardio-metabolic disease prevention through exercise training. *Sports Medicine* (Accepted)

95% contribution.

I wrote the literature review, created the figures and revised it.

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55% contribution.

I performed the experiments, analysed and interpreted data, wrote and revised the manuscript.

Paper 3 **Denham, J,** O'Brien, B.J., Prestes, P., Brown, N.J., & Charchar, F.J. (2016). Increased expression of telomere-regulating genes in endurance athletes with long leukocyte telomeres. Journal of Applied Physiology, 120(2): 148-158.

85% contribution.

I established the experimental design, conducted subject recruitment and testing, completed all laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Paper 4 **Denham, J.**, Marques, F.Z., O'Brien, B., & Charchar, F.J. (2014). Exercise: putting action into our epigenome. *Sports Medicine*, 44: 189-209.

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I wrote the literature review, created the figures and revised the manuscript.

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80% contribution.

I contributed to the experimental design, conducted subject recruitment and testing, completed laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Paper 6 **Denham, J.,** O'Brien, B.J., & Charchar, F.J. (2015). Genome-wide sperm dna methylation changes after three months of exercise training in humans. *Epigenomics*.

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I contributed to the experimental design, conducted subject recruitment and testing, completed laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Paper 7 **Denham, J.,** Marques, F.Z., & Charchar, F.J. (2014). Leukocyte telomere length variation due to DNA extraction method. *BMC Research Notes*, 7(1): 877.

75% contribution.

I contributed to the experimental design, conducted subject recruitment, completed laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Paper 8 **Denham, J**., Feros, S.A., & O'Brien, B.J. (2015). Four weeks of sprint interval training improves 5 km run performance. *Journal of Strength and Conditioning Research*, 29(8): 2137-2141.

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I contributed to the experimental design, conducted subject recruitment and testing, completed laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Paper 9 **Denham, J.,** Brown, N.J., Tomaszewski, M., O'Brien, B.J., & Charchar, F.J. Low augmentation index in endurance athletes: a role for cardiorespiratory fitness. (Under Review).

85% contribution.

I established the experimental design, conducted subject recruitment and testing, completed all laboratory experiments, analysed and interpreted the data, wrote and revised the manuscript.

Conference abstracts arising from my PhD candidature

A total of 13, including 6 international, 3 national and 5 University (Ballarat, Federation University, University of New England) conferences.

Denham, J, O'Brien, B.J., Prestes, P., Brown, N.J., & Charchar, F.J. (2016). Endurance exercise and physical activity: means to delay the end. Inaugral University of New England Postgraduate Conferece, Armidale, NSW, January 2016. (Invited presentation)

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Denham, J. Quarrel, S., Nield, A., Marques, A., O'Brien, B.J., & Charchar, F. Altered leukocyte mir-21 and -210 abundance after 4 weeks of sprint interval training (SIT). *6th Exercise and Sports Science Conference and Sports Dieticians Australia Update: Research to Practice*, Adelaide, Australia, March 2014. (Poster and oral)

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increase in telomere length of ultra-marathon runners. *International Convention on Science, Education and Medicine in Sport (ICSEMIS)*, Glasgow, Scotland, July, 2012. (Oral)

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Denham, J., Nankervis, S.A., O'Brien, B.J., Denniff, M., Ullah, S., Pascoe, D., Tomaszewski, M., & Charchar, F.J. The effect of ultra-marathon running on leukocyte telomere length. *Annual Federation University Australia Research Conference*, Ballarat, Australia, November, 2011. (Oral)

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Summary

Exercise training is one of the few therapeutic interventions that improves health span by delaying the onset of age-related diseases and preventing early death. Despite the clear benefits to health conferred by exercise training, our understanding of the underlying molecular mechanisms remain crude. The primary purpose of this thesis is to determine and analyse the molecular biology changes that occur with strenuous aerobic exercise. Specifically, the main objectives were to investigate the impact of strenuous aerobic exercise training on structural DNA modifications, measured in context with cardiovascular health and fitness adaptations.

In the first part of this thesis I investigated the influence of endurance exercise training on leukocyte telomere length and cardiovascular health. Leukocyte telomere length reflects biological age. Indeed, excessively short leukocyte telomeres are associated with age-related chronic diseases. Epidemiological studies indicate endurance athletes live longer than people from the general public who do not engage in extensive aerobic exercise training. In Chapter 2, my literature review on the subject of exercise and telomere biology suggested that, at the time of this study, the impact of exercise training on leukocyte telomere length was equivocal. Therefore, to determine whether strenuous aerobic exercise training influences biological ageing (assessed by leukocyte telomere length), I conducted two cross-sectional studies on leukocyte telomere length differences between endurance athletes and healthy controls.

The first study (Chapter 3) was a cross-sectional analysis of leukocyte telomere length between athletes and controls, determined by quantitative polymerase chain reaction (qPCR). This is a relative measurement of telomere length expressed as a telomere (T) to single copy gene (S) ratio. Relative to the healthy controls (n = 56), the ultra-marathon runners (n = 67)

possessed 11% longer leukocyte telomeres in age-adjusted analysis (ultra-marathon runners vs controls; average T/S ratio: 3.56 vs 3.16, $p = 1.4 \times 10^{-4}$) and the difference was not explained by the favourable cardiovascular health profile exhibited by the athletes ($p = 2.2 \times 10^{-4}$). The difference in leukocyte telomere length indicated the athletes had reduced their biological age by 16.2 years. To elucidate the potential mechanism for the longer leukocyte telomeres observed in endurance athletes, I recruited another cohort of athletes and controls and measured leukocyte telomere length and gene expression of genes involved in telomere length regulation. In the second study (Chapter 4), I describe data replicating the finding that endurance athletes possess longer leukocyte telomeres compared to healthy controls (athletes v controls mean T/S ratio ± SE: 3.64 ± 0.06 vs 3.38 ± 0.06, $p = 0.002$). This difference was associated with a concomitant increased activity of two important telomere regulating genes, telomerase reverse transcriptase (*TERT*) and adrenocortical dysplasia homolog (*TPP1*) (2-fold and 1.3-fold, respectively, both $p < 0.05$). The difference in leukocyte telomere length and leukocyte telomere-regulating gene (*TERT* and *TPP1* mRNA) expression was ameliorated after adjusting for maximal oxygen uptake and resting heart rate (all $p > 0.05$). This finding indicates that cardiorespiratory fitness is an important determinant of telomere biology. Together, these two cross-sectional studies suggest that regular endurance exercise training is associated with longer leukocytes telomeres and that this is likely achieved through higher *TPP1* and *TERT* mRNA expression gained through improved cardiorespiratory fitness. The findings in Chapters 3 and 4 provide evidence for extensive endurance exercise training as an effective lifestyle strategy to attenuate biological ageing.

In parallel to telomere length changes, epigenetic modifications (e.g. DNA methylation) caused by environmental factors alter the transcriptomic milieu of cells. My thorough literature review (Chapter 5) revealed that exercise training seems to rearrange chromatin by modifying the DNA methylome in a variety of cells and that the extent is dictated by exercise

duration and intensity. Therefore, in the second part of my thesis, I investigated the DNA methylation changes in leukocytes (which are somatic cells) and sperm (male germ cells) from healthy men before and after sprint interval training (SIT). Unlike traditional, long duration training at moderate intensity training, SIT involves short, intense (>85% $\dot{V}O_{2max}$ to supra-maximal) efforts followed by periods of rest (3–4 min), typically repeated 3–8 times. It is an effective type of training that improves cardiorespiratory fitness quicker than traditional long slow distance training. Thus, to establish the DNA methylome changes associated with SIT, I conducted two training studies and analysed the leukocyte and sperm methylomes using the Infinium HumanMethylation450 BeadChip (Illumina).

My third study (Chapter 6) provides the first evidence showing an association between DNA methylation changes paralleled with improvements to lipid profile and cardiorespiratory fitness in humans. Twelve young men (18–24 years) undertook SIT (thrice weekly) for four weeks. Resting blood samples were obtained and whole-blood leukocytes were isolated by red blood cell lysis. Genome-wide DNA methylation was assessed using the 450K BeadChip (Illumina). Cardiorespiratory fitness, determined by maximal oxygen uptake, was improved by 2.1 $ml \cdot kg^{-1} \cdot min^{-1}$ and low-density lipo-protein cholesterol was decreased by 3.9% after SIT ($p < 0.05$). Notably, the leukocyte methylome was significantly affected by SIT, in regions throughout the genome in relation to CpG islands – CpG islands, North shores, N shelves, South shores and South shelve – and the nearest genes – 3' untranslated region (UTR), 5' UTR, exonic, intergenic, intronic, non-coding and promoter regions (all $p < 0.001$). Genes with differentially methylated CpG sites ($q < 0.005$) after SIT were enriched for cardiovascular gene ontology (GO) terms that included metabolic activity, biological adhesion and antioxidant activity. Similarly, pathway analysis revealed genes involved in focal adhesion, calcium signaling and mitogen activated protein kinase were modulated by SIT-induced DNA methylation changes. Amongst the 205,987 probes relating 32,445

transcripts differentially methylated after SIT ($q < 0.05$), with methylation changes between 0.1 – 62.8%, the largest and most statistically significant demethylated site was in the epidermal growth factor (*EGF*) gene, causing decreased mRNA expression. As with *EGF*, the microRNA-21 and microRNA-210 genes (MIR21 and MIR210, respectively), known for their roles in cardiovascular disease (ischemic heart disease and coronary atherosclerosis), had modest but consistently statistically significant DNA methylation changes at numerous CpG sites, which altered mature microRNA abundance. Together, these data suggest that genome-wide DNA methylation changes occur after short-term intense exercise training concurrently with improvements to blood cholesterol profile and cardiorespiratory fitness.

The data presented in this thesis provided evidence that the epigenome of somatic cells is malleable to exercise. There is mounting evidence supporting the premise that environmental perturbations cause DNA methylation changes and these are subsequently transgenerationally inherited, altering phenotypes of future generations. In the current study I also asked the question; can exercise training reconfigure the DNA methylome of male germ cells (sperm)? Therefore, my next study (Chapter 7) entails an analysis of the impact that three months of SIT has on genome-wide DNA methylation of sperm in healthy men.

Thirteen subjects undertook twice-weekly SIT for three months, while the controls were asked not to change their current physical activity habits (if any). Sperm samples were donated before and after the three-month intervention. Mature sperm were isolated using density gradient centrifugation and DNA was extracted using the Purelink Genomic DNA Mini Kit (Life Technologies). Global and genome-wide DNA methylation was assessed using an enzyme-linked immunosorbent assay-based kit and the 450K BeadChip (Illumina), respectively. Relative to controls, the cases decreased their resting heart rate and had a higher maximal treadmill speed during exercise testing (both $p < 0.05$). Cases had decreased global DNA methylation after SIT compared to controls ($p < 0.05$). Genome-wide DNA methylation

analysis revealed numerous modest (0.3 – 6%) methylation changes to 7509 CpG sites, relating to 4602 transcripts ($q \leq 0.1$). Differentially methylated CpG sites were in genes associated with developmental biology, which included GO terms, such as developmental process, anatomical structure, embryonic morphogenesis and organ development, together with known pathways regulated by exercise training (MAPK, ErbB and PI3K-Akt signalling). Genes with increased methylation were associated with numerous human diseases, with most overrepresented being psychiatric disorders (schizophrenia, Parkinson's disease and autism). Notably, paternally imprinted genes associated with other diseases were also differentially methylated after SIT. Therefore, exercise training is associated with the modifications to genome-wide DNA methylation of both somatic and germ cells.

In conclusion, the studies presented as a series of peer-reviewed publications, outlines investigations that describe an influence of strenuous exercise training on leukocyte telomere length regulation and the DNA methylome of both leukocytes and germ cells. Both of these molecular changes in leukocytes and sperm provide evidence for novel molecular mechanisms by which exercise improves cardiovascular health and fitness. Future investigations should focus on longitudinal studies determining whether these changes are required for improved health and fitness, and should establish whether exercise-induced DNA methylation changes are transgenerationally inherited, and if so, what impact this has to future generations. Such discoveries could change national physical activity guidelines and policies, by emphasising the benefit of regular exercise both in the present and to future offspring.

Chapter 1 Background

Introduction

Cardiovascular disease is the biggest financial burden to the Australian health care system and kills the most Australians each year.[1] It is estimated that abolishing global physical inactivity alone would eliminate 6 to 10% of the world's non-communicable diseases, including type 2 diabetes, breast and colon cancer, and coronary artery disease.[2] Indeed, increasing physical activity is associated with a dose-dependent reduction in coronary artery disease risk.[3] The health benefits of exercise are not confined to primary prevention, but exercise training can halt and regress atherosclerotic lesions in coronary artery disease patients.[4,5] Furthermore, a superior cardiorespiratory fitness is associated with a reduced risk of heart failure-related mortality, regardless of the presence of other traditional cardiovascular disease risk factors, such as smoking, obesity, high blood pressure, a family history of cardiovascular disease and diabetes.[6] Despite the clear cardiovascular health benefits conferred by regular exercise training, the molecular mechanisms important for cardiovascular health and the prevention of premature death are poorly understood. The study of exercise training induced cardiopulmonary adaptations and analysis of telomere biology and epigenetic modifications may delineate how exercise training prevents, manages and attenuates cardiovascular disease and improves health span.

The thesis structure is presented in Figure 1. The following sections briefly discuss the biology of leukocyte telomere length and DNA methylation, as these topics are comprehensively covered in Chapters 2 and 5, respectively. *

*References for Chapter 1 are located in Volume 2, pages 442-454.

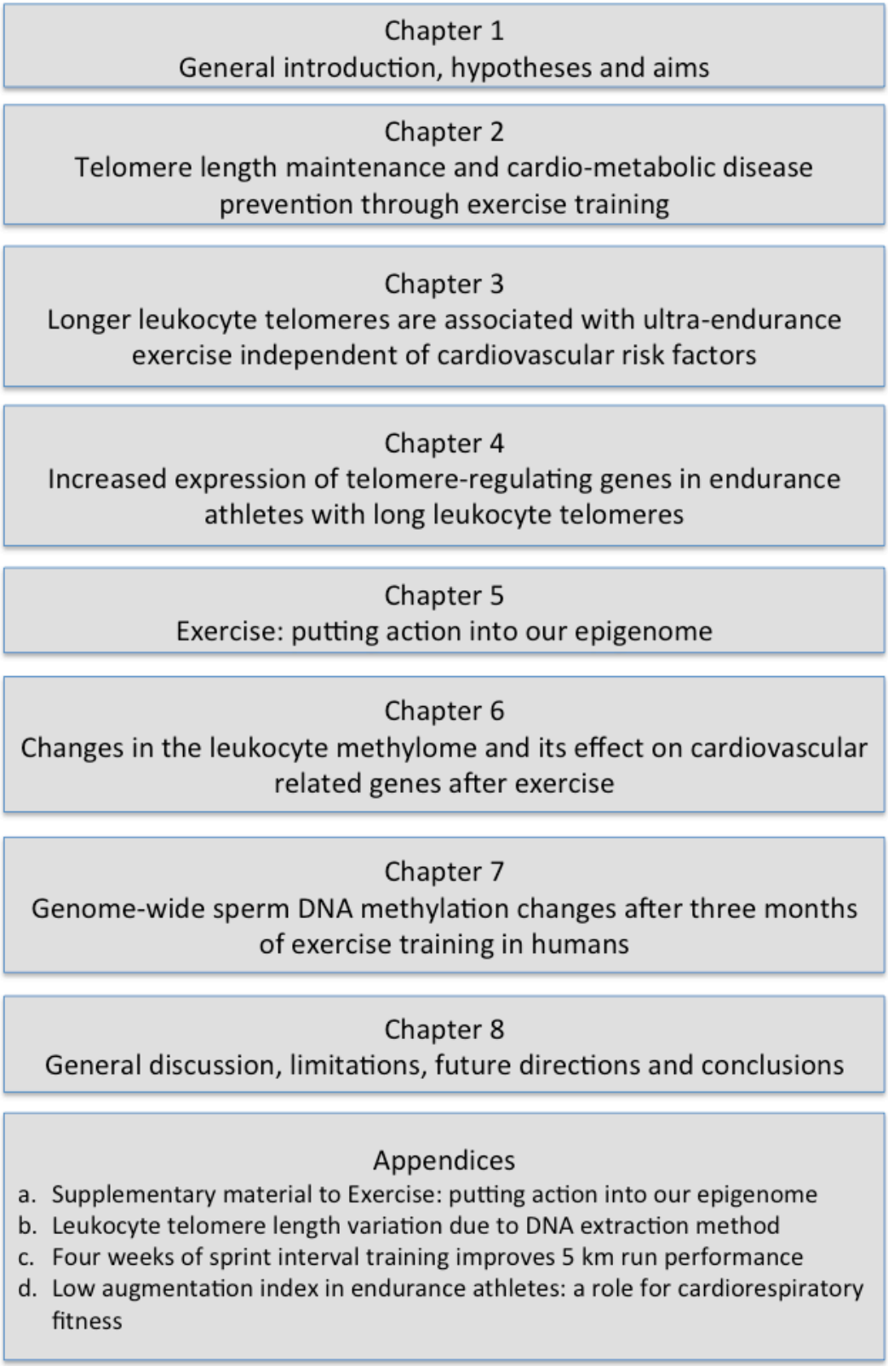

Figure 1. Thesis structure.

Telomere biology

Telomeres are repetitive DNA sequences (in mammals – 5'TTAGGG^{n}3') that prevent chromatin fusion events and maintain genomic integrity by capping chromosome ends.[7] In the absence of telomerase and due to the end replication problem, telomeres shorten during each round of cell division.[8,9] Once a critical length is reached, DNA damage pathways are triggered leading to cellular senescence,[10] which in turn decreases tissue vitality and accelerates organismal ageing. Therefore, telomere length is reflective of cellular mitotic history and leukocyte telomere length is a marker of biological age.[11]

Short leukocyte telomeres are implicated in the pathogenesis of age-related diseases, including coronary artery disease,[12-14] type 2 diabetes[15] and some cancers.[16,17] Leukocyte telomere length holds prognostic value for adverse coronary artery events[18,19] and is inversely correlated to mortality risk.[20-22] A comprehensive review of literature on telomere length in context with age-related cardio-metabolic disease and exercise training is found in the proceeding section, to introduce the reader to telomere biology (Chapter 2). Exercise training is typically associated with a reduced risk of age-related diseases, particularly coronary artery disease.[3] Therefore, it is possible that exercise partly prevents the pathogenesis of age-related diseases through telomere length maintenance.

Rationale for telomere and epigenetic exercise physiology studies on leukocytes

The majority of human studies have analysed telomere length in leukocytes. Most of the studies included in this thesis are on the analysis of telomere biology and DNA methylation in leukocytes. Therefore, a rationale for the study of leukocyte molecular exercise physiology is provided. There are several advantages of analysing telomere length and DNA methylation in whole-blood leukocytes: 1) they are easily collected by a relatively non-invasive, routine procedure; 2) leukocyte telomere length is correlated to that of other tissues, including

heart,[23] adipocytes, skeletal muscle and skin cells;[24] 3) leukocytes circulate through the body, come in contact with and are affected by numerous organs that are otherwise unobtainable from healthy subjects (brain, colon, lung, heart, kidney, stomach, etc). Their gene expression profile mimics that of the organs, making them sentinels of healthy/diseased tissues;[25,26] 4) Low-grade chronic inflammation is associated with ageing (referred to as, 'inflammaging') and age-related chronic diseases, where the immune system is well known to play an important role;[27-29] 5) The exhaustion of stem cell proliferative capacity is a detrimental phenotype of ageing.[30] The replicative history of leukocyte subsets, assessed by telomere length, mirrors that of hematopoietic stem cells;[31,32] and 6) hypertension is a risk factor of more severe and fatal cardiovascular diseases (e.g. myocardial infarction and stroke), and leukocytes are thought to be involved in the development of hypertension, through up-regulated toll-like receptor activity and inflammation.[33] Monocytes and macrophages are also directly involved in the pathogenesis of atherosclerosis,[34,35] making them an important tissue to study in context with cardiovascular health. Therefore, leukocytes are excellent biomarkers of disease and have a role in the pathogenesis of ageing and age-related chronic diseases. Considering the capacity of regular exercise training to lower circulating inflammation, improve immune function[36] and reduce blood pressure,[37] the aforementioned diseases and ageing may be partly prevented or attenuated by exercise-induced modifications to gene expression through telomere length and epigenetic mechanisms.

Epigenetics and DNA methylation

Epigenetics is the study of the change in gene expression independent of a change to DNA sequence. Epigenetic modifications encompass DNA (methylation, hydroxylation, etc) and histone modifications (methylation and acetylation), and depending on the definition used, may also include the change to gene expression regulated by other mechanisms (e.g. small non-coding RNAs – microRNAs, etc). MicroRNAs are 18–24 nucleotide RNA molecules

that degrade mRNA or block translation by binding to the 3' UTR of mRNA.[38] The studies outlined in my thesis primarily focused on DNA methylation. DNA methylation is the process whereby a cytosine neighbouring a guanine (dinucleotide) acquires a 'methyl' group – carbon with three hydrogen atoms bound – at the 5th carbon on the cytosine base.[39] This alters the electromagnetic charge causing conformational changes[40] to chromatin.[41] DNA methylation works in concert with other histone modifications and heterochromatin proteins to cause changes to gene expression by blocking DNA binding proteins and transcription factor binding sites.[42,43] Typically, DNA methylation is associated with transcriptional inactivation and activation when found in the gene promoter and gene body regions, respectively.[44] There is significant cross-talk between DNA methylation and telomere length as changes to either influences the other, to ultimately influence gene expression and complex phenotypes.[45-47]

DNA methylation and the environment

DNA methylation plays a crucial role in embryonic and cellular development, and is vulnerable to environmental changes.[40,42,48,49] A pioneering study revealed the DNA methylome is vulnerable to changes caused by lifestyle, through the analysis of monozygotic twins (individuals with identical genotypes).[50] In early life (3 years old) the monozygotic twins DNA methylation and histone modifications were indistinguishable, yet in later life (50 years old) their epigenomic profiles were discordant.[50] More recently, the DNA methylomes of patients with different age-related disease are beginning to be characterised,[51-55] allowing researchers to identify DNA methylation loci involved in lifestyle-related disease.

As previously stated, many of the age-related diseases with aberrant DNA methylation changes are somewhat preventable or manageable by physical activity.[56] Exercise training may attenuate the risk of developing or the progression of age-related diseases through the

modulation of epigenetic modifications, though experimental data is currently lacking. The reader is introduced the current paradigm of epigenetics and the influence of exercise on epigenetic modifications is discussed in a literature review in Chapter 5.[57]

Transgenerational epigenetic inheritance

Environmental-induced epigenetic modifications may be inherited across multiple generations (transgenerationally). Environmental toxins, plastics and endocrine disruptors administered to gestating rats caused ongoing dysfunctional DNA methylation changes with paralleled phenotype disturbances experienced through to the fourth generation of rats.[40,58] It is, however, currently unclear as to whether positive lifestyle choices, such as healthy diet or exercise, can cause adaptive changes to DNA methylation and health-related phenotypes. Rats on a low-protein diet have produced offspring with up-regulated hepatic gene expression and DNA methylation changes in genes involved in lipid and cholesterol metabolism, such as the key lipid regulator, peroxisome proliferator-activated receptor alpha (*PPARA*).[59]Although our current understanding of exercise-epigenetics is relatively primitive, this area of research has enormous potential to impact national physical activity policies and our understanding of best practice for the health of current and future generations.

Hypotheses

The hypotheses that are the basis of this thesis are as follows:

1) Regular endurance exercise training is associated with longer leukocyte telomeres, which in turn is associated with improved cardiovascular health risk factors including body mass index, blood pressure, cholesterol, triglycerides and circulating markers of inflammation (IL-6, CRP, leptin, ICAM-1, E-selectin).
2) Engagement in endurance exercise training will lead to differential expression of genes involved in telomere length regulation.

3) Sprint interval training (SIT) improves cardiovascular health and fitness by causing changes in leukocyte DNA methylation of genes and microRNAs that regulates cardiovascular physiology.
4) Exercise training contributes to changes in the human sperm DNA methylome in genes related to diseases.

Aims

The overarching aim of this thesis is to determine the impact of strenuous exercise training on leukocyte telomere, DNA methylation and corresponding gene expression changes measured in context with cardiovascular health and fitness in humans.

To fulfil this aim, I conducted two cross-sectional studies analysing leukocyte telomere length between endurance athletes and healthy controls. Furthermore, I conducted two training studies to determine whether the improvement to cardiovascular health and fitness was associated with changes to the genome-wide DNA methylation in human leukocytes and sperm.

The specific aims of this thesis were to:

1) Determine whether endurance exercise training is associated with longer leukocyte telomeres (Chapter 3).
2) Elucidate any differences in leukocyte telomere length and potential mediating cardiovascular risk factors between endurance athletes and healthy controls (Chapter 3 and 4).
3) Establish whether athletes also have differentially expressed genes important for telomere length regulation compared to controls (Chapter 4).
4) Identify whether particular physical activity, sedentary behaviour or cardiorespiratory fitness parameters mediate the telomere length and gene expression difference

between endurance athletes and controls, and whether these are correlated to telomere length and telomere-regulating genes (Chapter 4).

5) Establish the genome-wide leukocyte DNA methylation changes associated with four weeks of SIT in healthy young men, and if these changes are paralleled by improvements to cardiovascular health parameters and cardiorespiratory fitness (Chapter 5).
6) Identify if the SIT-induced changes in leukocyte DNA methylation are associated with gene expression changes in microRNA and or genes related to cardiovascular health and disease (Chapter 6).
7) Delineate the genome-wide DNA methylation changes in human sperm after three months of SIT, determine the genomic location of these changes and whether these are associated with genes involved in the pathogenesis of disease (Chapter 7).

Significance

Cardiovascular disease is the world's biggest health problem, contributing to 30% of all deaths.[60] Over three million physical inactivity-related deaths occur annually,[2] making physical inactivity the fourth deadliest killer.[61] A greater understanding of the molecular processes by which exercise improves cardiovascular health will provide crucial knowledge that will lead to discoveries that could significantly reduce the global cardiovascular disease burden.

Accumulating evidence indicates telomere shortening in the manifestation of cardiovascular disease.[13] Considering telomere elongation occurs through the recruitment and up-regulation of telomerase,[9,62,63] controlled, telomerase reactivation therapies or lifestyle strategies up-regulating telomerase activity may treat and prevent cardiovascular disease. In fact, cardiac-specific gene (telomerase reverse transcriptase, *TERT*) therapy administered after myocardial infarction improves cardiac function, telomere extension and life-span of mice.[64] Therefore, it

is vital to establish if exercise training attenuates telomere attrition and the underpinning molecular mechanisms. Understanding the molecular signals leading to improved cardiovascular health will one day allow the development of novel pharmaceuticals to mimic the impact of exercise. Given the eradication of physical labour in the workplace and the prevalence of physical inactivity,[61,65] a development such as the 'exercise pill' will be a vital development. Furthermore, molecular signatures associated with the exercise response or non-response will be useful for monitoring exercise training interventions and precision exercise prescription.

Some environmentally induced epigenetic modifications to one generation of mammals are trans-generationally inherited and affect the health of the offspring.[40,58,59,66] If exercise could favourably influence the epigenome and if these changes were trans-generationally heritable, then the ideal exercise that improves the health of future generations must be elucidated. Identifying the optimal type and amount of exercise training that confers the healthiest offspring would have the capacity to changes exercise and physical activity guidelines and policies, to ultimately decrease the burden of disease.

The results included in my thesis have provided evidence showing the capacity for aerobic exercise training to: 1) prevent telomere shortening and that this effect is partly due to cardiorespiratory fitness adaptations (higher maximal oxygen uptake and lower resting heart rate); 2) influence the leukocyte DNA methylome in conjunction with improved lipid profile and cardiorespiratory fitness; and 3) increase sperm methylation in genes related to common human diseases. Consequently, these proof-of-principle results may spawn future studies to determine the optimal frequency, intensity, time and type of exercise that improves human health span through telomere length maintenance and modulation of somatic and germ-cell epigenomes. If identified and translated into practice, this knowledge would spare the health

care system substantial financial constraints, and prevent disease-related hospitalisations and deaths. This, in turn, would improve the healthy living and ageing of the population.

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Telomere Length Maintenance And Cardio-Metabolic Disease Prevention Through Exercise Training

Short title: Exercise and Telomeres

Joshua Denham[1], Brendan J. O'Brien[2], Fadi J. Charchar[3,*]

[1]School of Science and Technology, University of New England, Armidale, NSW, Australia.

[2]Faculty of Health, Federation University Australia, University Drive, Mt Helen, Victoria, Australia.

[3]Faculty of Science and Technology, Federation University Australia, University Drive, Mt Helen, Victoria, Australia.

[*]Corresponding author: Fadi Charchar: email: f.charchar@federation.edu.au; phone: +613 5327 6098; fax: +613 5327 9242.

Key points

- Telomere shortening is a hallmark of ageing and is associated with a host of cardio-metabolic diseases prevented or managed by exercise training.
- Exercise training, a superior cardiorespiratory fitness and limited sedentary behaviour may prevent age-related cardio-metabolic disease through telomere length maintenance, though the molecular mechanisms responsible are incompletely understood.
- Future investigations are required to determine the optimal exercise prescription and the regulating factors – including epigenetic modifications, non-coding RNAs, oxidative stress and the telomere position effect – that avoid excessive telomere shortening to prevent premature biological ageing and disease.

Abstract

Telomeres are tandem repeat DNA sequences located at distal ends of chromosomes that protect against genomic DNA degradation and chromosomal instability. Excessive telomere shortening leads to cellular senescence and for this reason telomere length is a marker of biological age. Abnormally short telomeres may culminate in the manifestation of a number of cardio-metabolic diseases. Age-related cardio-metabolic diseases attributable to an inactive lifestyle, such as obesity, type 2 diabetes mellitus and cardiovascular disease, are associated with short leukocyte telomeres.

Exercise training prevents and manages the symptoms of many cardio-metabolic diseases whilst concurrently maintaining telomere length. The positive relationship between exercise training, physical fitness and telomere length raises the possibility of a mediating role of telomeres in chronic disease prevention via exercise. Further elucidation of the underpinning molecular mechanisms of how exercise maintains telomere length should provide crucial

information on how physical activity can be best structured to combat the chronic disease epidemic and improve the human health span. Here, we synthesise and discuss the current evidence on the impact of physical activity and cardiorespiratory fitness on telomere dynamics. We provide the molecular mechanisms with a known role in exercise-induced telomere length maintenance and highlight unexplored, alternative pathways ripe for future investigations.

1 Introduction

Telomeres are repetitive DNA sequences located at the ends of chromosomes that function to maintain genome integrity [67]. In most human cells, telomeres shorten with each round of cell division [68,69] and as such telomere length is reflective of cellular replicative history. Telomeres shorten with ageing and environmental stress, making telomere length a biomarker of biological age. Given the telomere length correlation and synchrony of telomere shortening between somatic cells, leukocyte telomere length is a useful biomarker of telomere lengths from other tissues [24].

Shorter average leukocyte telomere length is typically associated with age-related chronic diseases, such as cardiovascular disease [14,19,70], type 2 diabetes mellitus (T2DM) [15,71], some cancers [17], and psychological stress [72,73]. Furthermore, telomere dysfunction is involved in the pathogenesis of rare genetic diseases, called *telomere syndromes* (reviewed elsewhere [74]). Telomere shortening may also cause the manifestation of non-communicable, age-related diseases. Importantly, the aforementioned diseases associated with short leukocyte telomeres are partly prevented, managed or even regressed by regular physical activity [75-77]. The aim of this review is to discuss and synthesise the current literature on the impact of exercise on telomere biology and discuss potential mechanisms underlying how exercise influences telomere dynamics. Directions for future research will be provided but initially the reader will

be introduced to telomere biology and a brief review of leukocyte telomeres and cardio-metabolic disease is provided.

2 Telomere biology

2.1 Telomeres and telomere-associated proteins (shelterin)

Mammalian telomeres are repetitive stretches of non-protein coding DNA (5-TTAGGG-3_n) positioned at the distal ends of linear chromosomes (Figure 1a). Human leukocyte telomere length varies between ~5–15 kilobases (kb) [24,78] and shortens by approximately 20–50 base pairs (bp) yearly [79]. Telomeres shorten due to inability of DNA replication enzymes to copy the entire 3' end of the leading (G-rich) strand (Figure 1a) [80]. Furthermore, oxidative stress accelerates telomere attrition, with the telomeric DNA GGG triplet most vulnerable to reactive oxygen species [81,82], and antioxidant compounds attenuate telomere shortening *in vitro* [83]. It is currently unclear precisely how excess inflammation shortens telomeres. It could be that elevated inflammation exacerbates oxidative stress and promotes cellular turnover causing telomere shortening. Once telomere shortening has reached a critical length, telomere dysfunction ensues and signals cellular senescence through DNA damage pathways – ataxia telangiectasia mutated (ATM), ataxia telangiectasia and rad3 related (ATR), p21 and p53 [10,84,85]. This, in turn, decreases the proliferative capacity of the tissue leading to an aged phenotype and overall decrease in tissue vitality. There are, however, telomere-associated proteins that repress DNA damage signalling pathways by regulating telomere length; this is primarily accomplished through the formation of unique telomere complexes.

Six telomere-associated proteins (telomere repeat-binding factor 1 [TRF1], telomere repeat binding-factor 2 [TRF2], TRF1-interacting nuclear factor 2 [TINF2], adrenocortical dysplasia homolog [commonly referred to as TPP1], protection of telomeres 1 [POT1] and TRF2-interacting protein [TRF2IP]), collectively known as *shelterin*, bind to the telomeres and

regulate their length (reviewed in de Lange [84] and Nandakuma and Cech [63]) (Figure 1b and c). Telomeres and shelterin form unique structures (t- and d-loops [86] [Figure 1d]) and serve as protective caps at the ends of chromosomes to prevent chromosomal end-to-end fusion and allow the complete replication of protein-coding DNA. TRF1 and TRF2 are abundant telomere-binding proteins that play unique roles at the telomeres [87,88]. While TRF1 negatively regulates telomere length [89], TRF2 protects telomere-mediated chromosomal end-to-end fusion and cellular senescence [90]. TINF2 regulates and interacts with TRF1 and TRF2 [91]. TPP1 and POT1 bind to single stranded telomeric DNA (G-strand overhangs), and interact to recruit and increase the processivity of telomerase resulting in telomere elongation [92,93]. TRF2 and POT1 independently protect telomeres from DNA damage response pathways (ATM and ATR, respectively) and inevitably prevent cellular senescence through telomere length maintenance [94]. TRF2-interacting protein (TRF2IP) (alternatively known as RAP1) binds indirectly to telomeric DNA through its interaction with TRF2 and contributes to the prevention of telomere fusion events [95,96]. RAP1, however, is dispensable as mice lacking a functional *Rap1* gene are viable, unlike mice null for other shelterin components (*Trf1* or *Tpp1*) [97]. RAP1 also has extra-telomeric functions, as RAP1 silences sub-telomeric genes, but also acts as a transcription factor through interactions with other genomic locations [98]. Therefore, shelterin aid in the stabilisation of telomere length, avoids adverse chromosomal events and represses the activation of DNA damage response signalling pathways at telomeres via the formation of t- and d-loops [99,100]. Although not within the scope of this review, it is important to note telomeres have nucleosomes and histone proteins that are vulnerable to epigenetic modifications [46]. Furthermore, sub-telomeric DNA is heavily methylated and telomere shortening is observed with accompanying epigenetic modifications required for open, transcriptionally active, chromatin [45,101]. Open telomere chromatin may subsequently up-regulate telomere transcription to produce telomeric repeat-containing RNA

(TERRA). TERRA is a long non-coding RNA molecule that inhibits telomerase by binding to the telomerase RNA template through its complementary and repetitive RNA sequence (5'-UUAGGG-3') [102]. The impact of exercise training on TERRA is currently unknown. Thus, epigenetic modifications and TERRA are added tiers of telomere length regulation. Nonetheless, progressive telomere shortening occurs in the absence of the enzyme *telomerase*.

2.2 Telomerase

Telomerase is a reverse-transcriptase ribonucleoprotein capable of adding telomeric repeats to DNA [9]. In humans, telomerase is made up of two main proteins (telomerase reverse transcriptase [TERT] and telomerase RNA component [TERC]) and additional accessory proteins (dyskeratosis congenita 1 [DKC1] and NOP10 ribonucleoprotein [NOP10]) (Figure 1d). Although telomerase activity is high in human ovaries and testis [103], somatic cells have variable telomerase activity. For example, leukocyte subsets express low to moderate levels of telomerase activity [104], yet telomerase is extremely low or undetectable in human skeletal myocytes [103,105]. Conversely, telomerase activity is up-regulated in most human cancers and the majority of cancer cells have short telomeres [104,106,107]. Remarkably, *in vitro* experiments involving the reintroduction of telomerase to human cells previously lacking telomerase activity extends telomeres and extends cellular life span [69,108]. Thus, the rate of somatic cell telomere shortening that occurs during ageing is influenced by the proliferative rate of cells, shelterin and telomerase activity.

The regulation of telomerase activity is a complex dynamic process, discussed in detail elsewhere [109,110]. While POT1 and TPP1 recruit telomerase to telomeres and increase telomerase processivity – the capacity for telomerase to add telomeric DNA [93,111] – many other non-telomere-associated proteins also govern telomerase activity. Telomerase up-regulatory factors modulated by exercise (acute or chronic) include Akt, insulin-like growth

factor 1 (IGF1) and interleukin 6 (IL6), amongst others [109,112]. Interestingly, accumulating evidence indicates a role for telomerase in the regulation of biological pathways outside that of telomere biology [98,113].

2.3 Alternative lengthening of telomeres

Alternative lengthening of telomeres (ALT) is another mechanism with the capacity to elongate telomeres independent of telomerase. ALT may occur through telomere sister chromatid exchanges or homologous recombination-dependent DNA replication [114,115]. Indeed, 10–15% of cancers lack telomerase activity and rely on ALT for telomere length maintenance. Whereas ALT has been reported largely in a relatively small subset of cancers [116-118], recently ALT activity was observed in normal mouse cells *in vivo* [119]. Common mouse strains, however, have significantly longer telomeres (10 to 80 kb) [120,121] and the induction of cellular senescence is different compared to humans [122]. Nonetheless, ALT may be a normal part of telomere length regulation and whether exercise training influences telomere length through ALT is currently unknown.

3 Telomeres and cardio-metabolic diseases

Regular physical exercise is not only associated with the decreased risk of developing age-related cardio-metabolic diseases (i.e. obesity, T2DM and heart disease), but exercise training also attenuates and manages particular disease symptoms [56,123,124]. In this section, we discuss telomere length in context with cardio-metabolic diseases that exercise training is known to prevent or manage. While not discussed here, it is important to emphasise shorter average leukocyte telomeres are found in individuals with elevated psychological stress [72,73,125] and those consuming poor diets [126,127] – lifestyle factors that contribute to an increased risk of many cardio-metabolic diseases.

3.1 Obesity

Most studies on leukocyte telomere length and obesity-related phenotypes – (e.g. body-mass index [BMI], weight and waist circumference) [128-133] – show inverse relationships, though some do not [134,135]. Childhood obesity has been associated with shorter leukocyte telomeres in both sexes [136], and in boys only [137]. There are reported sex differences, suggesting the adverse effect of obesity-related phenotypes on leukocyte telomere length may be more apparent in women than men [130,138]. These inconsistencies may be due to the different races and ages of participants studied. Recently, data from weight-loss interventions involving dietary amendments with or without additional physical activity and counselling services support the relationship between leukocyte telomere dynamics and the regulation of obesity-related phenotypes. For instance, increased leukocyte telomere length was correlated with reduced body weight, BMI and waist circumference in middle-aged to older adults (n = 521) after a five-year Mediterranean dietary intervention [129]. A shorter (two-month) intensive lifestyle intervention elicited weight-loss with leukocyte telomere lengthening in adolescents [139]. The reduction in obesity phenotypes caused by the dietary interventions are consistent with the premise that telomere shortening elicits a causal influence on obesity-related phenotypes and that lifestyle strategies aimed at lengthening telomeres may effectively combat disease.

3.2 Type 2 diabetes mellitus

Patients with T2DM have shorter leukocyte telomeres compared to their non-diabetic counterparts [15,140-143] and the extent of telomere shortening is dependent on patient outcomes. T2DM patients with a history of myocardial infarction (MI) exhibited shorter leukocyte telomeres than those without a history of MI and healthy controls [142]. Additionally, leukocyte telomeres are progressively shorter in T2DM patients with more diabetic complications (such as retinopathy, incipient nephropathy and cardiovascular disease) [141]. The genetic predisposition to elevated oxidative stress could contribute to telomere shortening in T2DM

patients. The uncoupling protein 2 (*UCP2*) gene codes a protein important for the electron transport chain and down-regulates reactive oxygen species (ROS) production. T2DM patients with the functional variant allele *UCP2* –866A exhibit shorter (~170 bp) leukocyte telomeres compared to their peers homozygous for the G allele, suggesting a genetic contribution to ROS-induced telomere attrition [15]. Further, leukocyte telomere length shortening over 10 years was related to increases in BMI, insulin, glucose and homeostasis model assessment of insulin resistance [144]. Whereas excessive telomere shortening in T2DM patients is established, whether leukocyte telomere length holds predictive value for T2DM risk is unknown, as two large prospective studies have yielded conflicting results [145,146].

3.3 Cardiovascular disease

Consistent with T2DM, the presence of cardiovascular disease is characterised by short leukocyte telomeres relative to healthy individuals [147-150]. Accelerated biological ageing is shown in patients with atherosclerosis, such that their leukocyte telomeres are ~300 bp shorter than healthy controls; a difference equating to 8.6 years' worth of telomere shortening [70]. Chronic heart failure patients not only had shorter leukocyte telomeres than age and sex-matched controls, but the extent of telomere shortening was dependent on plaque formation; incrementally shorter telomeres were observed in patients with one, two or three atherosclerotic manifestations, defined as coronary, cerebrovascular and peripheral vasculature, or any combination [151]. Shorter leukocyte telomeres are associated with coronary artery calcification in a cardiovascular disease-free, middle-aged population, suggesting that the leukocyte telomere shortening observed in atherosclerotic diseases may not be a consequence of the disease [14]. The shortened leukocyte telomeres (~300 bp) observed in MI patients indicated patients were biologically 11.3 years older than their age-matched healthy controls [19]. When patients were divided in telomere length quartiles, those with the shortest telomeres had a 3-fold increased risk for MI [19]. Notably, these findings were supported by

some [152] but not others [18]. In a large cohort (n >1500), risk of developing coronary heart disease was increased in subjects in the lowest tertile of telomere length and was attenuated by statin treatment [13]. Whilst the current literature suggests short telomeres are predictive of future coronary heart disease, the predictive ability of telomere length for cerebrovascular disease is unclear [153]. Leukocyte telomere length was not associated with ischemic stroke, in a cohort of 259 males who were initially free from disease that progressed to develop ischemic stroke [154]. The predictive value of leukocyte telomeres in disease prognosis may be limited to middle-aged subjects and not older adults (>75 y) [155], yet older individuals (60 to 97 years) with shorter telomeres have poorer survival due largely to infectious diseases and heart disease [20]. While short baseline telomere length was associated with increased cardiovascular disease-related mortality risk in women, telomere shortening over 2.5-years was associated with greater mortality from cardiovascular disease in men aged 70 to 79 years [156].

3.4 Is telomere shortening a cause or consequence of cardio-metabolic disease?

Considering the available human research, it is important to note that it is unclear as to whether the leukocyte telomere shortening in cardio-metabolic diseases is a cause or consequence of disease. Evidence from mouse and *in vitro* experiments support the former. *Terc*$^{-/-}$ mice, without detectable telomerase activity, exhibit short telomeres and age-related phenotypes – splenic and intestinal atrophy, reduced body weight, haematological abnormalities, reduced proliferative potential, hair loss and tumour formation [157-159]. *Terc*$^{-/-}$ mice from mixed genetic backgrounds also suffer from hypertension [160], depleted haematopoietic progenitor cells [161] and T2DM [162]. Endothelial dysfunction caused by telomere-mediated cellular senescence in *Terc*$^{-/-}$ mice was restored after treatment with antioxidant compounds [163]. Low-grade chronic inflammation and oxidative stress are common symptoms and contribute to the pathogenesis of age-related cardio-metabolic

diseases [164-167]. Both inflammation [168] and oxidative stress [82,169] cause telomere attrition *in vitro*.

Telomere shortening alters the expression of neighbouring – subtelomeric – genes [170,171] and genes separated by large distances (10 kb) [172], a concept known as the *telomere position effect*. The change in gene expression may not be due to the telomere shortening *per se* but rather a loss of shelterin proteins or telomere chromatin formations [173]. Therefore, telomere shortening may contribute to the manifestation of age-related disease by deregulating transcription of particular genes throughout the genome. Indeed, older versus younger human fibroblasts have shorter telomeres and increased expression of genes involved in chromatin modifying proteins [174], suggesting telomere shortening may facilitate chromatin remodelling through coordinated expression of chromatin modifying genes. Moreover, senescent human fibroblasts with shorter telomeres have differentially expressed sub-telomeric genes compared to quiescent fibroblasts [175].

A genome-wide association study (GWAS) found seven loci containing candidate genes (*TERC*, *TERT*, *NAF1*, *OBFC1* and *RTEL1*) were associated with leukocyte telomere length and risk of multiple cancers, idiopathic pulmonary fibrosis and coronary artery disease in humans, supporting a causal role of telomere shortening in the development of cardio-metabolic diseases [176]. Leukocyte telomere attrition rate over a 7 to 11-year time-period was inversely related to atherosclerosis progression, indicated by carotid intima thickness (cIMT) [177]. Furthermore, subjects with cIMT regression exhibited telomere elongation [177], indicating telomere lengthening is protective of vascular damage. Accordingly, compared to subjects with telomere maintenance or elongation after six years, those with telomere shortening had higher cIMT and an increased risk of cardiovascular events, independent of traditional cardiovascular disease risk factors [178]. Similar associations whereby telomere elongation is observed with attenuated disease symptoms – or vice versa – have been observed in T2DM

[144] and obese [129,139] patients. Although mouse and human studies support the concept that telomere shortening may facilitate disease manifestation and progression, it is important to emphasise other unmeasured factors may mediate telomere and disease dynamics. Lifestyle factors such as physical activity, diet and psychological stress are potential confounding factors that could influence telomere length and disease risk independently. These factors could also facilitate a pro-inflammatory or oxidative cellular environment which could, in turn, promote telomere attrition, cellular senescence and disease.

Importantly, the cardio-metabolic diseases related to telomere shortening are somewhat preventable and managed by physical activity [75-77]. The prevention or management of cardio-metabolic disease conferred by exercise training could be partly driven by telomere length maintenance. Alternatively, exercise training could prevent disease through a reduction in disease-related risk factors (blood pressure, lipid profile, adiposity, etc.), systemic inflammation and oxidative stress, or psychological stress to ultimately prevent telomere shortening.

4 Telomere biology and physical activity

Regular engagement in exercise training is associated with longer telomeres and may attenuate telomere attrition. The optimal exercise recommendations for telomere length maintenance, however, remain elusive. Research has involved the analysis of telomere length in context with physical activity habits, predominantly assessed by physical activity questionnaires, case-control studies involving habitual exercisers and less-active or sedentary controls, and or physical fitness measures (i.e. maximal oxygen consumption [$\dot{V}O_{2max}$] or metabolic equivalent of task [METs]). In the next section, we discuss findings on the effect of physical activity and fitness on leukocyte telomere dynamics mainly in leukocytes and skeletal muscle (Table 1).

4.1 Telomere length and physical activity

Physical activity was positively correlated to leisure-time physical activity levels in 2401 twin volunteers [179]. The leukocyte telomeres were 200 bp longer in individuals who performed the most physical activity compared to those who engaged in the least amount [179]. Moreover, in twins discordant for physical activity levels, leukocyte telomeres were 80 bp longer in the twin engaging in the most physical activity [179]. Supporting these results, a linear relationship between physical activity and telomere length was observed in a cohort of female nurses [180], suggesting that physical activity may be protective of telomere length.

There may be a threshold for the amount of physical activity ideal for telomere length maintenance. An inverted-U relationship was observed when 69 healthy subjects were divided into estimated energy expenditure quartiles, with those expending moderate amounts of energy exhibiting the longest peripheral blood mononuclear cell (PBMC) telomeres [181]. Strikingly, longer leukocyte telomeres and a lower percentage of short telomeres were found in men who engaged in moderate physical activity compared to men who engaged in low or high amounts of activity 29 years prior to telomere length assessment [182]. Despite supporting an inverted-U relationship between physical activity and telomere length [181], physical activity was assessed from self-reported questionnaires [182] which often do not yield valid or reliable results. There are also some studies that do not show any statistically significant associations between physical activity and leukocyte telomere length [183-185]. Interestingly, recreational physical activity may be more important for telomere maintenance as job-related physical activity was not associated with leukocyte telomere length in 981 individuals (aged 45–84 y) [186]. Physical fitness, however, was not measured in these studies and the lack of association between telomere length and physical activity may be partially explained by the physical activity assessment methods used.

Habitual or regular exercise training seems to protect against telomere shortening, particularly in postmenopausal women. In cross-sectional analyses, postmenopausal women who performed 60 min of resistance and aerobic exercise more than three times per week for an average of 19 months exhibited significantly longer PBMC telomeres compared to their sedentary peers [187]. Postmenopausal women with stage I-III breast cancer, who participated in moderate to vigorous physical activity also have significantly longer PBMC telomere length compared to their sedentary counterparts [188], suggesting exercise intensity may be an important determinant of telomere length. Conversely, recent data indicates that sedentary behaviour is detrimental to leukocyte telomere length. In a large epidemiological study, screen-based sedentary behaviour was inversely associated with leukocyte telomere length in 6405 adults (20 – 84 y) [189]. Consistent with these findings, telomere lengthening was observed in older adults who reduced their sitting time, after a six-month intervention involving prescribed exercise and counselling [190].

4.2 The impact of age on telomere length and physical activity

The positive effects of physical activity on telomere maintenance may be restricted to certain age groups. In older adults (≥65 y, n = 2006) physical activity assessed by the Physical Activity Scale for the Elderly [191], was not associated with leukocyte telomere length. Rather than physical activity, physical capacity may have a greater impact on telomere length maintenance in older adults. For example, gait speed [192] and Barthel index [193,194] – an assessment of ability to perform activities of daily living, were positively correlated to telomere length in older adult populations. In a cohort of 548 same-sex Danish twins, leukocyte telomere length increased with every unit increase in physical ability score [195]. Moreover, a 10 year longitudinal study indicated a decline in grip strength was related to a faster leukocyte telomere attrition, potentially caused by elevated circulating inflammatory markers (interleukin-1β and cortisol) [196]. Sit to stand performance and walking distance were

associated with longer leukocyte telomeres in a cross-sectional analysis involving 582 older adults, while estimated energy expenditure and time to complete five chair stands were associated with attenuated telomere attrition over a 5-year period, suggesting physical activity and performance are important for telomere maintenance even in later years of life [197].

Duration of physical activity may not be as important as the intensity of exercise for younger individuals. One study involving 667 adolescent subjects revealed physical activity, objectively assessed by accelerometers worn for seven days, was unrelated to leukocyte telomere length [198]. Telomere length was, however, positively associated with exercise intensity [198], providing evidence that vigorous exercise may be protective of telomeres in young individuals.

4.3 Endurance exercise, cardiorespiratory fitness and telomere length

Endurance athletes have a superior level of cardiorespiratory fitness, resulting from prodigious amounts of aerobic exercise training. They also have a much lower risk of cardiovascular and cancer related mortality, and live longer than sedentary individuals [199]. Interestingly, telomere attrition was attenuated in middle-aged (~51 y) German track and field athletes who ran an average of 80 km a week, such that they had similar PBMC telomere lengths compared to younger (~21 y) athletes and sedentary controls [200]; findings that have been supported by others [201]. Ultra-endurance athletes running an average of 40–100 km per week possessed 11% longer leukocyte telomeres compared to healthy subjects, indicating these athletes had prevented approximately 16 years' worth of age-related telomere shortening [202]. Of note, we recently verified our previous findings that endurance athletes have longer leukocyte telomeres and attenuated biological ageing [202] in another cohort of endurance (triathletes, long distance runners and cyclists) subjects, such that the athletes possessed longer telomeres than controls and had prevented 10.4 years of telomere shortening caused by ageing [203]. In a much smaller (athletes n = 17, controls n = 15) study, however,

athletes running ~33 km a week had similar granulocyte and lymphocyte telomeres to that of sedentary controls [204]. The discrepancy maybe due to the leukocyte subsets analysed or method of telomere measurement – quantitative fluorescent in situ hybridization (qFISH). Therefore, the majority of available literature finds strenuous, endurance exercise training is associated with longer telomeres. The adherence to regular exercise training may be required in order to prevent telomere shortening as former Finnish elite athletes possessed comparable leukocyte telomere lengths to that of their sedentary counterparts [205]. It is currently unclear as to why telomere length seems to be somewhat maintained in these athletes, but there is mounting evidence indicating a role for cardiorespiratory fitness.

Maximal oxygen uptake was an independent predictor of leukocyte telomere length in a cohort of 57 young (21 y) and older (~62 y) adults, and explained 20% of the overall variance in leukocyte telomere length [201]. Others have also demonstrated weak to moderate ($r = 0.11$ to 0.44) positive correlations between leukocyte telomere length and VO_{2max} [201,206]. Leukocyte telomere length was ~169 bp longer in stable chronic heart failure patients with high (> 7 METs) fitness compared to their unfit (< 5 METs) peers [207]. In a large epidemiological study including 1764 US adults, predicted maximal oxygen uptake was associated with longer telomeres, such that individuals with moderate (39.1 $ml \cdot kg^{-1} \cdot min^{-1}$) or high (50.9 $ml \cdot kg^{-1} \cdot min^{-1}$) cardiorespiratory fitness possessed longer leukocyte telomeres than those with poor fitness (30.7 $ml \cdot kg^{-1} \cdot min^{-1}$). The positive relationship between VO_{2max} and telomere length is not exclusive to leukocytes, as skeletal muscle telomeres are longer in subjects with higher VO_{2max} [208]. Thus, improvement in cardio-respiratory fitness seems an important adaptation potentially involved in telomere length maintenance caused by exercise training.

It is important to emphasise that while ample evidence indicates that endurance exercise is associated with longer leukocyte telomeres in athletes compared to less-active controls, data on skeletal muscle telomeres is not as consistent. Unlike highly proliferative leukocytes,

skeletal muscle telomeres are quiescent and the limited available data demonstrate recreational physical activity does not cause excessive telomere shortening in young or older adults [209]. Skeletal muscle telomeres may not succumb to chronological ageing. Conversely, the increased oxidative stress associated with ageing may facilitate telomere shortening, as older mobile adults have reduced reactive oxygen species and longer leukocyte and thigh skeletal muscle telomeres compared to their immobile peers [210]. Endurance athletes, however, with exercise-associated fatigue possess short muscle telomeres [211]. Healthy athletes have been documented to have longer [208] or comparable [212] muscle telomere lengths relative to sedentary controls. Resistance exercise-trained athletes have borderline significantly longer minimum and mean telomeres compared to controls [213]. Therefore, current data on endurance exercise and resistance training suggest that endurance exercise is not detrimental to leukocyte or muscle telomere length maintenance, though there could be an upper limit to the protective effect of exercise, considering shorter muscle telomeres were inversely correlated to maximal strength [213] and training history (years and hours spent running) [212].

4.4 Longitudinal, prospective studies on lifestyle changes and leukocyte telomere length

Well-controlled lifestyle interventions are beginning to provide insights on the impact of exercise training on the age-associated telomere attrition. A three month intervention consisting of a healthy diet (high in natural food products), stress management, social support and 30 minutes of walking exercise six days a week significantly increased PBMC telomerase activity in low-risk prostate cancer patients, who refused traditional cancer treatment [214]. After a 5-year follow-up, the experimental group had modestly longer telomeres, an effect mostly unobserved in the controls [215]. Whilst these preliminary findings propose a healthy lifestyle may increase telomerase activity and telomere length the study was small (cases n = 10, controls n = 25) and some cases in the lifestyle intervention

experienced telomere shortening comparable to the controls. Similar findings were reported in a group of 59 middle-aged sedentary males after a much shorter (6-month) moderate-intensity exercise training intervention [216], although a control group was not included. In a large, 12-month randomised controlled trial, women in either the control (n = 87), exercise-only (n = 117), calorie-reduced diet only or exercise (n = 117) and calorie-reduced diet (n = 118) groups showed a similar, minimal change in leukocyte telomere length that was not statistically significant [206]. Therefore, it is premature to claim whether exercise training can solely prevent telomere shortening or induce telomere elongation. Additional controlled exercise training studies involving different exercise prescription will be required to identify whether exercise alone can prevent age-associated telomere shortening.

Evidence indicates psychological stress is detrimental to telomere length maintenance [72,73]. Importantly, the negative association between psychological stress and leukocyte telomere length was abolished in postmenopausal women achieving the Center for Disease Control and Prevention's recommended physical activity levels (75 min of vigorous exercise per week) [217]. Strikingly, over a one year follow-up period, postmenopausal women who experienced more adverse life stressors experienced greater telomere attrition (~35 bp decrease for every life stressor), however telomere attrition was attenuated by a healthy lifestyle (more physical activity, better sleep quality and a healthier diet) [73]. When telomere attrition and physical activity levels were analysed independently, a one standard deviation below or at the group average was associated with a significantly faster telomere attrition [73]. Thus, exercise training may combat psychological stress-induced telomere shortening.

Taken together, physical activity seems to confer a beneficial effect on leukocyte telomere length maintenance as shown by association studies. Despite positive correlations between physical activity levels and leukocyte telomere length, the ideal exercise prescription for telomere conservation is not yet established. Endurance athletic status is associated with

longer leukocyte telomeres predominantly in middle-aged subjects. No evidence indicates extensive aerobic exercise is associated with shorter telomeres in healthy subjects. The inconsistencies throughout the literature are likely due to genetic diversity of subjects, age ranges, DNA extraction methods [218], methods of assessing physical activity and telomere length quantitation [219-221], and the controlling of other confounding factors (eg. diet, psychological stress and socioeconomic status). Researchers should control for the aforementioned confounding factors to attempt to isolate the impact exercise training has on telomere biology. Notably, it is unclear as to whether individuals who inherited and were born with long telomeres have increased cardiorespiratory fitness and consequently engage in more exercise training. Indeed, there is a genetic propensity for $\dot{V}O_{2max}$ [222] and the training-induced responsiveness to $\dot{V}O_{2max}$ [223], making it plausible that physical activity data may be purely associational and that genetic influence programs telomere maintenance. It is suggested that literature on telomere length and physical activity assessed solely by self-reported physical activity questionnaires should be interpreted with caution as they have inherent issues such as recall-bias and over/under-estimation of actual physical activity levels is common [224-226]. Whereas the benefits of aerobic exercise on telomere length are commonly reported, data on the effects of resistance training on telomere length are scarce. It is recommended future research should consider quantifying cardiorespiratory fitness in context with self-reported physical activity and telomere dynamics. Additional randomised, controlled exercise training interventions and longitudinal telomere length assessment with healthy and clinical populations will help discern the involvement of telomere regulation on disease management and regression. Further research is also required to elucidate the ideal exercise prescription required for telomere length maintenance in other unexplored tissues. This knowledge in turn can be used to optimise the physical training required to combat cellular ageing and prevent age-related cardio-metabolic disease.

5 Molecular mechanisms in exercise-induced telomere dynamics

5.1 Telomerase activity

Physical activity and exercise training are associated with longer telomeres but the molecular mechanisms underpinning the relationship are not very well understood. The ability of telomerase to extend telomeres and prevent telomere shortening-induced cellular senescence [9,69] suggests a crucial role for increased telomerase activity mediating telomere length consequential to exercise training. Indeed, elevated telomerase activity after chronic exercise training has been demonstrated in a variety of tissues, including human PBMCs [214,216], mouse skeletal muscle [227], heart [228], aorta [200] and hippocampi [229]. Endurance athletes who regularly engage in rigorous aerobic exercise exhibit a 2.5- and 1.8-fold increased telomerase activity compared to young and middle-aged sedentary subjects, respectively [200]. Despite the capacity of chronic exercise training to influence telomerase activity, whether an acute bout of exercise has a similar effect remains to be explored. The exercise training-induced increase in telomerase activity is dependent on TERT and endothelial nitric oxide synthase (eNOS) protein expression, as long-term exercise training does not modulate telomerase activity in *Tert*$^{-/-}$ or *eNos*$^{-/-}$ mice [228]. Mice treated with recombinant growth hormone or IGF1 exhibited an 8- and 14-fold increase in cardiac telomerase activity, indicating these growth factors are mediators for the up-regulation telomerase activity in cardiac myocytes of exercised mice [228]. These data provided crucial evidence elucidating a role for growth hormone, IGF1, eNOS and TERT in the up-regulation of telomerase activity induced by exercise training.

5.2 Shelterin expression

Telomerase must be recruited to telomeres for telomere elongation to occur; a process tightly regulated by shelterin proteins [63,110]. 30 minutes of aerobic exercise at 80% of peak oxygen consumption (VO_{2peak}) significantly up-regulates leukocyte *TRF2IP* and *TERT* expression in healthy young men [230]. Whereas acute aerobic exercise modulates some telomere-associated

genes in leukocytes, this may not effect protein abundance. For example, PBMC *TRF1*, *TRF2* and *POT1* expression were all up-regulated the day following a seven-day ultra-marathon race in eight athletes, but this did not translate into increased protein abundance or telomerase activity [231]. MicroRNAs (miRNAs) are small RNA molecules that negatively regulate translation or degrade messenger RNA (mRNA) targets, in turn, limiting protein abundance. The acute exercise-induced decrease in expression of the miR-96 and -186-targeted transcript *TERF2IP* was observed with a concomitant increase in miRNA molecules [230]. miRNA regulation of shelterin genes is a possible explanation for the unchanged protein abundance observed previously [231]. Whilst seven-days of intense endurance exercise may not facilitate shelterin protein abundance, the overall adaptation to endurance exercise may lead to altered shelterin protein expression. 57 endurance-trained athletes showed increased PBMC *TF2* mRNA and protein levels, with increased telomerase and longer telomeres compared to sedentary subjects [200]. Similarly, aortic [200] and heart [228] *TRF2* mRNA and protein levels are increased in mice after three weeks of voluntary wheel running. Therefore, some shelterin proteins particularly TRF2, are modulated by long-term exercise training in PBMCs, heart and aortic tissue. There is, however, limited evidence showing the acute effects of exercise on shelterin expression.

Relative to other tissues, skeletal muscle shelterin may not be as responsive to exercise training. In athletes, no changes were observed in skeletal muscle *TRF1*, *TRF2* or *POT1* expression after a seven-day ultra-marathon [231]. However, the age-related increase in TRF1 protein expression in plantaris muscle of sedentary mice was attenuated in exercised mice [227], possibly through p38 mitogen activated protein kinase (MAPK) inactivation [232]. Considering TRF1 is a negative regulator of telomerase activity [110], the decreased TRF1 expression observed in conjunction with increased telomerase activity [227] may be a mechanism to counteract the telomere shortening observed in the exercised mice. TRF1 protein expression

is decreased after acute exercise in mouse skeletal muscle [232], but whether this is detrimental or occurs in conjunction with increased telomerase activity is currently unknown.

5.3 Other mechanisms

Exercise training prevents many age-related metabolic diseases including cardiovascular disease [233], T2DM [234] and obesity [235], and is reflected by a longer telomere phenotype. Conversely, age-related chronic diseases are associated with shorter mean leukocyte telomere length [236] and are all characterised by low-grade chronic inflammation and oxidative stress [237]. Cell culture experiments indicate the former accelerates telomere shortening [168]. Oxidative stress also induces telomere attrition [83,238], with the telomere GGG sequence particularly vulnerable to damage caused by reactive oxygen species [82]. Importantly, regular exercise training reduces both low-grade chronic inflammation and circulating oxidative stress [36,239]. We, however, found no correlations between long-term exercise training, telomere length and inflammatory cytokines (interleukin-6, intercellular adhesion molecule-1 e-selectin, C-reactive protein and leptin) [202]. Additionally, a six month exercise training intervention in obese subjects improved antioxidant enzyme activity but did not alter leukocyte telomere length [240]. Therefore, it is difficult to ascribe telomere shortening to oxidative stress or inflammation *in vivo*. The study of other oxidative/inflammatory compounds and longer exercise training regimes may help elucidate whether exercise attenuates telomere shortening via reduced ROS and inflammation.

Small non-coding RNA, such as miRNAs are ~22 bp in length and serve as negative regulators of translation by targeting the 3' untranslated region (UTR) on mRNA target transcripts or through mRNA degradation. While shelterin gene and protein expression have been studied in context with exercise training, little is known about the miRNAs regulating these. Data from our laboratory demonstrated decreased TERF2IP expression with increased miR-98 and miR-186 abundance after acute exercise training [230]. In breast cancer tissue miR-

155 is up-regulated and down-regulates TRF1 protein abundance consequently promoting genomic instability [241]. Thus, the miRNA and other non-coding RNA, such as long non-coding RNA (including subtelomeric transcribed RNA, TERRA [242]), regulation of shelterin and telomere length changes associated with exercise is likely. Interestingly, miR-290-dependent regulation of retinoblastoma-like 2 protein which in turn impacts DNA methyltransferase (DNMT) enzyme activity and subsequent DNA methylation modulates telomere length and recombination [243]. To that end, telomere dynamics may also be influenced by epigenetic modifications and TERRA expression.

A change in gene expression without a change to the genetic sequence is the definition of epigenetic regulation. Epigenetic regulators such as DNA methylation and histone modifications are crucial for biological development and aberrant epigenetic profiles are associated with many age-related diseases [244,245]. The impact of exercise training on genome-wide DNA methylation is beginning to be delineated and has been discussed in detail elsewhere – [57,246-248]. The *TERT* gene has a cytosine neighbouring a guanine dinucleotide (CpG) island (a region abundant in CpGs) located in its promoter region regulating telomerase expression in telomerase-positive cells [249]. There are, however, conflicting data on the effects of DNA methylation in the proximal promoter region of *TERT* in immortal and other cell lines [250]. It will be important to identify the influence methylation status has on TERT expression in leukocytes from healthy human donors. Furthermore, the chromatin remodelling protein, CCCTC-binding factor, binds to exon 1 of *TERT* and represses *TERT* mRNA expression, highlighting the complex epigenetic regulation of *TERT* [251]. DNA methylation in concert with histone acetylation acts to negatively regulate *TERT* expression [252]. Work from the Blasco laboratory has provided insight into the interactions between telomere length and epigenetic modifications [46,101,253]. Telomere shortening in *Terc*$^{-/-}$ mice is accompanied by histone methylation and acetylation changes at telomeric and subtelomeric

regions, consistent with a more open, transcriptionally active chromatin [46]. DNMT-null mouse embryonic stem cells show exceptionally elongated telomeres, potentially due to telomere recombination events [101]. Thus, there is ample evidence supporting a role for epigenetic regulation in the exercise-induced changes to *TERT* and the expression of other telomere-associated genes, though this remains to be experimentally demonstrated.

Therefore, it is likely that telomere length is tightly controlled by shelterin-mediated telomerase recruitment in context with the cellular proliferative activity, which would undoubtedly be accelerated by increased oxidative stress and inflammation. Considering inflammation and oxidative stress shorten telomeres *in vitro*, it is possible that exercise may attenuate telomere attrition by maintaining a beneficial redox status and preventing chronic low-grade inflammation. Whilst only beginning to be delineated, the role of epigenetic modifications, chromatin structures and non-coding RNA, particularly miRNA and TERRA, molecules may serve as fine-tuners of shelterin and telomerase activity. The aforementioned molecular and cellular factors could be responsible for the telomere maintenance in individuals routinely engaging in aerobic exercise training or accelerated telomere attrition observed in sedentary individuals (Figure 2).

6 Conclusions

The prevention of age-related cardio-metabolic diseases is an adaptation conferred by engagement in regular exercise training, but the underlying molecular mechanisms are to date poorly understood. Cellular ageing through telomere shortening is attenuated by physical activity and exercise training yet the most favourable exercise prescription that maintains telomere length will require future study. The analysis of telomere length, shelterin expression and telomerase activity with exercise training interventions involving different exercise prescription including mode, frequency, duration and intensity, are warranted. Further, the impact of resistance training on telomere length is relatively unknown and

deserves attention. Efforts should be made to establish additional molecular pathways by which exercise maintains telomere length. Analyses on oxidative stress, inflammation, epigenetic modifications, non-coding RNAs (miRNAs and TERRA) and the telomere position effect are recommended. These studies will help shed light on telomere-mediated healthy ageing and disease prevention through exercise training, to aid exercise prescription recommendations.

Compliance with ethical standards

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Conflicts of interest

Joshua Denham, Brendan O'Brien and Fadi Charchar declare that they have no conflicts of interest relevant to the content of this review.

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Figure Captions

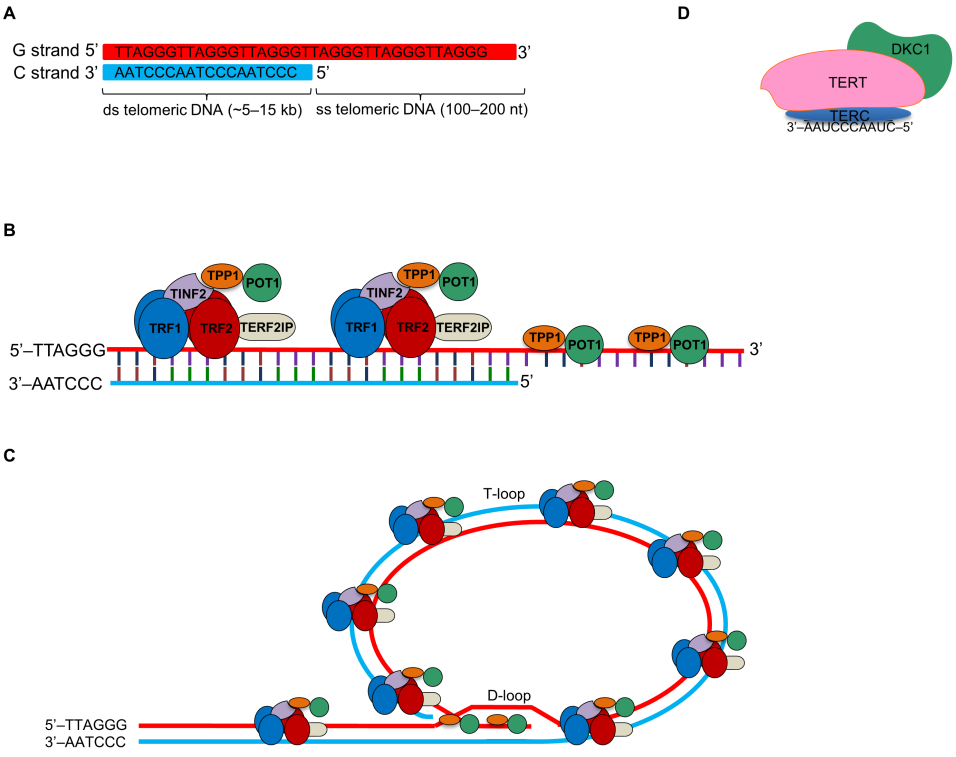

Figure 1. Telomeres, shelterin and telomerase. Telomeric DNA can form multiple structures that prevent genomic recombination and fusion events. (a) Telomeres are comprised of the double (ds, leading strand) and single-stranded (ss, lagging strand) DNA sequence – 5'-TTAGGG-3' and 3'-AATCCC-5', respectively. While ds telomere DNA varies between 5 and 15 kilobases (kb) depending on age, disease status and lifestyle factors, ss telomeric DNA is usually between 100 and 200 nucleotides (nt) long. (b) Ds and ss telomeric DNA are bound by telomere-associated proteins collectively known as *shelterin*. TRF1 and TRF2 bind directly to ds telomeric DNA. TINF2 and TPP1 bind POT1 to TRF1 and TRF2. TERF2IP binds to TRF2. TPP1 and POT1 also bind to ss telomeric DNA. (c) Telomeres also form circular structures called t- and d-loops that prevent the abrupt ending of telomeric DNA and DNA damage response pathways. (d) In humans, *telomerase* is formed by the TERT protein and RNA molecule, TERC. The TERC RNA has a complementary sequence to telomeric DNA – 3'-AAUCCC-5' – and serves as a template for telomere elongation through a reverse transcription process. TRF1, telomere repeat-binding factor 1; TRF2, telomere repeat-binding factor 2; TINF2, TRF1-interacting nuclear factor 2; TPP1, adrenocortical dysplasia homolog; POT1, protection of telomeres 1; TERF2IP, TRF2-interacting protein; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase.

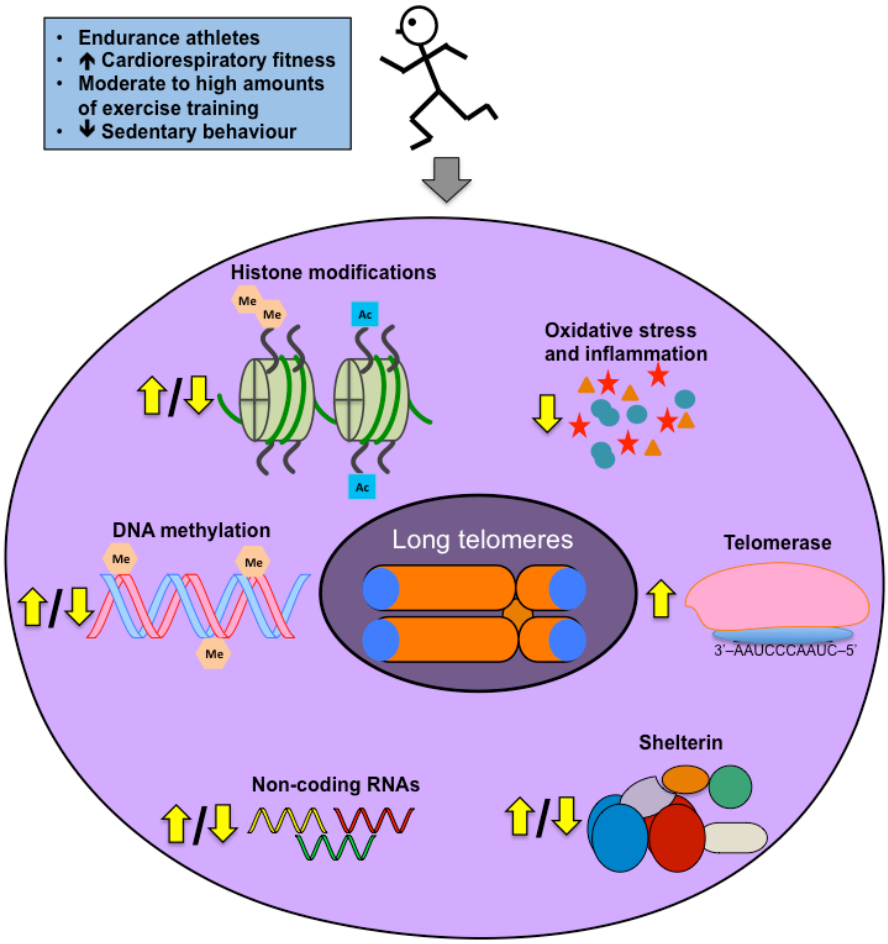

Figure 2. Mechanisms of telomere length maintenance conferred by exercise training.

Exercise training potential facilitates telomere length maintenance through many molecular mechanisms. Conversely, a sedentary lifestyle or lack of exercise training may deregulate the telomere maintenance pathways. Telomere length is likely regulated by epigenetic modifications including histone modifications (methylation [me] and acetylation [ac]) and DNA methylation. Non-coding RNAs such as miRNA may target shelterin mRNA to negatively regulate protein abundance and TERRA inhibits telomere elongation by binding to the TERC RNA template of telomerase competing against the telomeric DNA. Differential

regulation of shelterin expression and telomerase activity would regulate telomere length. Finally, inflammation and oxidative stress shortens telomeres *in vitro* and are candidates for telomere shortening *in vivo*. The yellow arrows indicate whether the particular variable is increased or decreased, or possibly both – depending on the location or interaction with other factors – in context with exercise training. mRNA, messenger RNA; miRNA, microRNA, TERRA, telomeric repeat-containing RNA.

Table 1 Current literature on exercise and telomere biology

Cell type	Participants[a,b,c,d,e]	Extraction method	Telomere measurement	Main result/s	Reference
	Athlete/control				
Leukocytes	61 endurance athletes and 61 healthy controls, M + F, ~30, VO_{2max} test and IPAQ.	Purelink Genomic DNA Mini Kit	qPCR	Endurance athletes exhibited longer leukocyte telomeres and increased *TERT* and *TPP1* mRNA expression compared to controls.	Denham et al. [203]
Buccal cells	20 endurance athletes and 42 sedentary controls, M + F, ~45.	QIAmp DNA Mini Kit	qPCR	Longer telomeres in athletes compared to controls.	Borghini et al. [254]
Leukocytes	392 former Finnish elite athletes and 207 controls, M, ~71, questionnaire.	?	qPCR	Former Finnish athletes have comparable age-adjusted telomere length compared to controls.	Laine et al. [205]

Leukocytes	67 ultra-marathon runners and 56 healthy controls, M, 43 ± 9.2.	?	qPCR	Ultra-marathon runners have 11% longer (16 years biologically younger) leukocyte telomeres compared to controls.	Denham et al. [202]
Lymphocytes and granulocytes	17 marathon runners and 15 sedentary controls, M + F, 54 ± 4, VO_{2max} test.	n/a	FISH	Similar telomere lengths between marathon runners and control. No statistically significant correlation between telomere length and VO_{2max}.	Mathur et al. [204]
Skeletal myocytes	5 young and older endurance athletes and healthy controls, M, young 24 and old 69, VO_{2max} test.	GenElute Mammalian Genomic DNA Miniprep Kit	qPCR	Older but not young endurance athletes have longer telomeres compared to their peers. Strong, positive correlation between VO_{2max} and telomere length.	Osthus et al. [208]

Skeletal myocytes	18 runners and19 sedentary controls, M + F, ~40.	Phenol/ chloroform	Southern Blot	Similar minimum and average telomere length between runners and sedentary controls. Moderate, inverse correlations between training history (years spent running and training hours) and minimum telomere length.	Rae et al. [212]
Leukocytes	27 (10 young and 17 older) endurance-trained individuals and 30 (15 young and older) sedentary controls, M + F, young 22 ± 1 and older 63 ± 2, VO_{2max} test.	?	Southern Blot	Older but not young, endurance-trained individuals had significantly longer leukocyte telomeres compared to their sedentary peers by ~900 bp. Moderate, positive correlation between VO_{2max} and telomere length.	LaRocca et al. [201]

Mononuclear cells Lymphocytes and granulocytes	57 (32 young and 25 older) endurance-trained individuals and 47 (26 young and 21 older) sedentary controls, M + F, young 21 and old 51, VO_{2max} test.	QIAmp DNA Blood Mini Kit	FISH and qPCR	Older sedentary individuals exhibited shorter mononuclear cell telomeres compared to all other groups. Up-regulated mononuclear cell telomerase activity in athletes (young and old) compared to sedentary controls.	Werner et al. [200]
Skeletal myocytes	7 power lifters and 7 sedentary controls.	Phenol/ chloroform	Southern Blot	A trend between higher minimum and average telomere length in power lifters compared to sedentary controls. Strong, inverse correlations between lower leg strength (squat and deadlift 1 repetition maximum) and minimum telomere length.	Kadi et al. [213]

Skeletal myocytes	13 FAMS and 13 healthy athletes, M + F, young, ~42 ± 11, VO_{2max} test.	Phenol/ chloroform	Southern Blot	Shorter minimum telomere length in FAMS compared to healthy athletes .	Collins et al. [211]
	Physical activity				
Leukocytes	6405, M + F, 20 – 84, questionnaire.	?	qPCR	Dose-dependent positive association between telomere length and physical activity levels.	Loprinzi et al. [255]
Leukocytes	582 older adults, M + F, 73 ± 5, modified Minnesota Leisure-Time Activities Questionnaire, timed 15-ft walk, chair stands and grip strength.	?	Southern Blot	Superior chair stand performance and greater walking distance associated with longer telomeres. Increasing physical activity and chair stand performance was associated with less telomere attrition in prospective analyses.	Soares-Miranda et al. [197]

Leukocytes	6405, M + F, 20 – 84, questionnaire.	?	qPCR	Every additional hour of screen-based sedentary behaviour is associated with 7% increased chance of possessing short telomeres.	Loprinzi [189]
Leukocytes	4576, M + F, ~55, binary.	Qiagen Blood Kit	qPCR	Long telomeres are associated with physical activity (at least 4 $hr \cdot week^{-1}$). Change in telomere length over 10 years is unaffected by physical activity.	Weischer et al. [256]
Leukocytes and skeletal myocytes	36 (12 young, 12 old-mobile and 12 old-immobile), M + F, young (25 y) and old (87.5 y).	QIAmp DNA Mini Kit	qPCR	Relative to old-immobile subjects, mobile older adults have attenuate oxidative stress and age-related telomere shortening in thigh muscle and leukocytes.	Venturelli et al. [210]

Leukocytes	239, F, 50-65, Stanford Brief Activity Scale.	QIAmp DNA Mini Kit	qPCR	Life-stress associated telomere attrition is attenuated by increased physical activity levels.	Puterman et al. [73]
PBMC	392, F, 62 ± 10, IPAQ.	?	Southern Blot	Postmenopausal breast-cancer patients who engage in moderate to high amounts of physical activity had longer telomeres than those not engaging in any physical activity by ~270 bp.	Garland et al. [188]
Leukocytes	204, M, 76, questions.	Gentra PureGene Blood Kit	Southern Blot	Compared to those engaging in low and high amounts of physical activity, those with self-reported moderate physical activity levels had the longest telomeres with less proportion of short telomeres.	Savela et al. [182]
Leukocytes	981, M + F, 45–84, Job Content Questionnaire	?	qPCR	No association with job-related physical activity.	Fujishiro et al. [186]

Leukocytes	5862 nurses, F, 58.7, questionnaire.	QIAmp 96 spin Blood Protocol	qPCR	Physical activity not associated with telomere length. Positive relationship between healthy lifestyle (non-smoking, moderate alcohol consumption, healthy body weight, moderate to high physical activity) and telomere length.	Sun et al. [257]
Leukocytes	44, F, 57.4 ± 5.6, card scanning.	G-spin Genomic DNA Extraction Kit	qPCR	Postmenopausal women regularly exercising (60 min, >3days/week) have longer telomeres than sedentary women.	Kim et al. [187]
Leukocytes	895, M + F, 33–79, three-point scale.	QIAmp	qPCR	Exercise frequency is an independent predictor of leukocyte telomere length . A healthy lifestyle explained 40% of the association between intelligence and leukocyte telomere length.	Kingma et al. [258]

Leukocytes	7813 nurses, F, 43 - 70, questionnaire.	QIAmp 96-Spin Protocol	qPCR	Longer telomeres in nurses who engaged in moderate or high amounts of physical activity, and moderate to vigorous intensity activities.	Du et al. [180]
Leukocytes	667 healthy adolescents, M + F, 14 – 18, accelerometer.	?	qPCR	Positive association between vigorous physical activity and telomere length in girls.	Zhu et al. [198]
Leukocytes	2284 nurses, F, 30 – 55, questionnaire.	QIAmp 96 DNA Blood Kit	qPCR	No association between physical activity and telomere length.	Cassidy et al. [184]
Leukocytes	63 healthy postmenopausal women, F, 62, questions.	Puregene DNA Isolation System	qPCR	Regular physical activity ameliorates the negative relationship between psychological stress and telomere length.	Puterman et al. [217]

Leukocytes	82, M + F, 18 – 80, questions.	Puregene Blood Core Kit	qPCR	No correlation between exercise and telomere length.	Song et al. [183]
Leukocytes	318 healthy individuals, M + F, 51.3, questions.	Gentra Puregene Blood Kit	qPCR	No interaction between telomere length and exercise and the presence of coronary artery calcium.	Diaz et al. [185]
Leukocytes	612 advanced prostate cancer patients and 1049 controls, M, 55 – 74, ?	QIAmp 96 DNA Blood Kit	qPCR	No correlation between physical activity and telomere length. Positive correlation between a healthier lifestyle (physical activity, diet, low BMI and low or no cigarette smoking) and telomere length.	Mirabello et al. [259]

Leukocytes	2006, M + F, 72 ± 5, PASE.	?	qPCR	No association between physical activity and telomere length.	Woo et al. [260]
PBMC	69, M + F, 60 ± 4.9, YPAS.	PureGene DNA Isolation System	qPCR	Moderate estimated energy expenditure associated with longest telomeres compared to low and high energy expenditure.	Ludlow et al. [181]
Skeletal myocytes	16 young and 26 older recreationally-active individuals, M + F, young 25 ± 4 and old 74 ± 4.	Phenol/ chloroform	Southern Blot	Similar minimum and average telomere lengths between young and older recreationally-active individuals.	Ponsot et al. [209]

Leukocytes	2401 white twins, M + F, 18 – 81, Allied Dunbar National Fitness Survey.	?	Southern Blot	Strong, positive relationship between physical activity and telomere length. More active twin had longer (~88bp) telomeres. Most active individuals had longer (~200bp) telomeres than their inactive peers.	Cherkas et al. [179]
Physical fitness					
Leukocytes	1764, M + F, 20 – 49, submaximal treadmill test.	?	qPCR	Compared to individuals with poor predicted cardiorespiratory fitness (30.7 $ml \cdot kg^{-1} \cdot min^{-1}$), those with moderate (39.1 $ml \cdot kg^{-1} \cdot min^{-1}$) or high fitness (50.9 $ml \cdot kg^{-1} \cdot min^{-1}$) possessed longer leukocyte telomeres.	Loprinzi [261]

PBMC	2488, M, middle age, vertical jump test, handgrip test, sit to stand and spirometry test.	?	qPCR Universal STELA	Number of short telomeres (<750 bp) are inversely associated with maximum jump height.	Maynard et al. [262]
Leukocytes	277 individuals, M + F, 76, grip strength.	QIAmp DNA Maxi Kit	qPCR	Faster telomere attrition associated with the decrease in grip strength after a 10-year follow-up.	Baylis et al. [196]
Leukocytes	117 elderly Koreans, F, 42 ± 0.7.	G-spin Genomic DNA Extraction Kit	qPCR	Medium, positive correlation between gait speed (6m) and telomere length.	Lee et al. [192]

Leukocytes	548 same-sex twins, M + F, 78.3, Physical Ability Score and five-point scale.	?	Southern Blot	Positive associations between physical ability score, physical activity and leukocyte telomere length.	Bendix et al. [195]
Leukocytes	44 Japanese patients, M + F, 74, physical assessment.	?	Southern Blot	Physical capacity associated with greater proportion of long telomeres (>9400bp) in females.	Maeda et al. [193]
Leukocytes	944 coronary heart disease outpatients, M + F, ~67, questions.	?	qPCR	Linear relationship between exercise capacity (maximum METs obtained during treadmill testing) and telomere length. Self-reported physically active individuals have longer telomeres (~73bp) than their inactive peers.	Krauss et al. [207]

Leukocytes	23 Japanese cerebrovascular disease patients, F, 77.7 ± 6.4, physical assessment.	?	Southern Blot	Physical capacity associated with greater proportion of long telomeres (>9400bp).	Maeda et al. [194]
A single bout of exercise					
Buccal cells	20 endurance athletes and 42 sedentary controls, M + F, ~45	QIAmp DNA Mini Kit	qPCR	Relative to basal, athletes had significantly shorter telomeres mid-way and at completion of an ultra-marathon trail race.	Borghini et al. [254]
Leukocytes T lymphocytes	22, M, 24 ± 7.	n/a	n/a	Acute, high-intensity exercise modulates leukocyte *TERT*, *SIRT6*, *TERF2IP* mRNA and microRNA (miR-15a, -96, -181 and -186) abundance.	Chilton et al. [230]

PBMC Skeletal myocytes	8, M + F, 44 ± 2.	Promega Wizard SV Kit	qPCR	Telomere-associated gene mRNA was increased in PBMC (*TERF1*, *TERF2*, *POT1*, *KU70* and *KU80*) and skeletal (*KU70* and *KU80*), without changes to protein content after a seven-day ultra-marathon race. No change to *TERC* and *TERT* mRNA abundance, telomere length or telomerase activity.	Laye et al. [231]
T lymphocytes	9 moderately trained (VO_{2max}: 56.9 ± 5.1), M, 26 ± 6.7.	?	qPCR	Acute, high-intensity exercise increases CD8+ T-cell telomere length, but does not alter CD3+ or CD4+ T-cell telomere length.	Simpson et al. [263]

PBMC	10 young (median age: 23) and 10 older (median age: 78) physically active individuals, M + F.	Qiagen Blood Kit	Southern Blot	Average telomere length decreases after acute exercise in young (PBMC and CD8+) and older (CD4+) individuals.	Bruunsgaard et al. [264]
	Training studies				
PBMC	59, M, 45–65, VO_{2max} test, ?	?	Southern Blot	Longer telomere and elevated telomerase activity after six months of exercise training.	Melk et al. [216]

Leukocytes	49 sedentary and overweight older adults, M + F, 68 y, IPAQ, 6-month intervention involving counseling and prescribed exercise.	?	qPCR	Reduced sitting time correlated to telomere lengthening.	Sjögren et al. [190]
PBMC	10 cases and 25 controls with prostate cancer, M, ~62.5, n/a, walking 30 min 6 days/week for 3 months (healthy diet, stress	?	qPCR	Longer telomeres after a 5-year lifestyle intervention.	Ornish et al. [215]

	management, meditation and counseling).				
Leukocytes	439 overweight/obese, F, 58, VO_{2max} test, 45 min of moderate to vigorous (70-80% HR_{max}) exercise 5 days/week for 1 year (with or without a calorie-reduced diet).	Qiagen Midi Kit	qPCR	Weak, positive correlation between telomere length and VO_{2max}. No change to telomere length after 12-month intervention.	Mason et al. [206]

Leukocytes	190 cases and 188 controls, M + F, ~55, n/a, increase physical activity.	Salting out method	qPCR	Lifestyle intervention did not have an effect on telomere length after a 4.5 y follow-up.	Hovatta et al. [265]
PBMC	24 prostate cancer patients, M, 62.2 ± 7.5, n/a, walking 30 min 6 days/week for 3 months (healthy diet, stress management, meditation and counseling).	n/a	n/a	Increased telomerase activity after lifestyle intervention.	Ornish et al. [214]

Leukocytes	16 obese individuals, F, 46.8 ± 6.4, VO_{2max} test, 60 min of treadmill walking/running @ 60% VO_2 reserve thrice weekly for 3 months.	Wizard Genomic DNA Kit	qPCR and Southern Blot	No change to telomere length after exercise moderate intensity exercise training.	Shin et al. [240]

Rodent studies

Hippocampus	45 Sprague-Dawley rats, M, pups, n/a, voluntary wheel running.	AllPrep DNA/RNA Mini Kit	qPCR	Voluntary wheel running prevented telomere elongation associated with maternal separation – a model of developmental stress.	Botha et al. [266]
Skeletal myocytes	22 C57BI/6 mice, F, 6 weeks old, n/a, 30 min of treadmill running @ 65% of V_{max}.	n/a	n/a	Acute exercise decreases *Trf1* expression in conjunction with increased p38-MAPK phosphorylation. No changes to other shelterin gene expression.	Ludlow et al. [232]

Liver, skeletal and cardiac myocytes	CAST/Ei J mice (6 young 8-week old, 11 1 year old sedentary and 10 1 year old exercised), M + F, 8 weeks and 1 year old, voluntary wheel running.	PureGene DNA Isolation System	qPCR	Voluntary wheel running attenuates cardiac myocyte and hepatocyte telomere length attrition, but accelerates skeletal myocyte telomere shortening. Up-regulated telomerase activity in skeletal myocytes after voluntary wheel running. Voluntary wheel running attenuated the age-related reduction in expression of telomere-associated genes (*Trf1*, *Trf2* and *Pot1*) in skeletal and cardiac myocytes but not hepatocytes.	Ludlow et al. [227]

Hippocampus	4 nestin-green fluorescent protein (expressing) and 12 C57BI/6, ?, n/a, 10 days of voluntary wheel running.	n/a	FISH	Voluntary wheel running restored lowered and further increased telomerase activity in a mouse-model of schizophrenia and controls, respectively. No change to telomere length after voluntary wheel running.	Wolf et al. [229]
Aortic and spleen-derived mononuclear cells	C57BI/6 wild-type and *Tert*$^{-/-}$ and *eNos*$^{-/-}$ mice, ?, n/a, n/a, voluntary wheel running for either 3 weeks or 6 months.	n/a	FISH	Increased telomerase activity, Trf1 and Trf2 expression, with decreased pro-apoptotic proteins (Chk2, p16 and p53) after 3 weeks of voluntary running in wild-type, but not *Tert*$^{-/-}$ and *eNos*$^{-/-}$ mice. No change to aortic telomere length after voluntary running.	Werner et al. [200]

Cardiac myocytes and leukocytes	C57Bl/6 wild-type and *Tert*$^{-/-}$ and *eNos*$^{-/-}$ mice,?, n/a, n/a, voluntary wheel running for either 3 weeks or 6 months.	n/a	FISH	While Tert and Trf2 protein expression and telomerase activity were up-regulated, pro-apoptotic proteins (Chk2, p16 and p53) were ameliorated after 3 weeks and 6 months of voluntary running in wild-type, but not *Tert*$^{-/-}$ and *eNos*$^{-/-}$ mice. Left ventricular myocyte and leukocyte telomere length unchanged after voluntary running.	Werner et al. [228]

[a] number and type of participants; [b] sex; [c] average age ± standard deviation or range; [d] method of assessing physical activity/estimated energy expenditure/exercise/fitness; [e] exercise training (other lifestyle interventions).

M = male; F = female; n/a = not applicable; qPCR = quantitative polymerase chain reaction; FISH = fluorescence in situ hybridization; STELA = single telomere length analysis; VO_{2max} = maximal oxygen consumption; bp = base-pairs; PBMC = peripheral blood mononuclear cell; TERT = Telomerase reverse transcriptase; TPP1 = adrenocortical dysplasia homolog; SIRT6 = Sirtuin-6; TERF2IP = Telomere repeat binding factor 2 interacting protein; IPAQ = International Physical Activity Questionnaire; PASE = Physical Activity Scale for the Elderly; YPAS = Yale

Physical Activity Survey; HR_{max} = heart rate maximum; $^{-/-}$ = deficient (gene); FAMS = fatigued-athlete myopathic syndrome; Vmax = maximal treadmill speed; eNos, endothelial nitric oxide synthase; BMI = body mass index; MET = metabolic equivalent of task; MAPK = mitogen-activated protein kinase; Pot1 = protection of telomeres-1; Trf = telomere-repeat binding factor; ? = unknown.

Chapter 3 Longer leukocyte telomeres are associated with ultra-endurance exercise independent of cardiovascular risk factors – published in *PLoS ONE*, 2013.

Longer leukocyte telomeres are associated with ultra-endurance exercise independent of cardiovascular risk factors

Joshua Denham[1], Christopher P. Nelson[2], Brendan J. O'Brien[1], Scott A. Nankervis[1], Matthew Denniff[2], Jack T. Harvey[1], Francine Z. Marques[1], Veryan Codd[2], Ewa Zukowska-Szczechowska[3], Nilesh J. Samani[2], Maciej Tomaszewski[2], Fadi J. Charchar[1,2*]

[1] School of Health Sciences, University of Ballarat, Mt Helen, Australia, [2] Department of Cardiovascular Sciences, University of Leicester and National Institute for Health Research (NIHR) Leicester Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester, LE3 9QP, [3] Department of Internal Medicine, Diabetology and Nephrology, Medical University of Silesia, Zabrze, Poland

**Corresponding author*: Prof Fadi Charchar, Room 228, F Building, Oppy Drive, University of Ballarat, Mt Helen, 3350. Ph: (03) 5327 6098, Fax: (03) 5327 9602, Email: f.charchar@ballarat.edu.au

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Abstract

Telomere length is recognized as a marker of biological age, and shorter mean leukocyte telomere length is associated with increased risk of cardiovascular disease. It is unclear whether repeated exposure to ultra-endurance aerobic exercise is beneficial or detrimental in the long-term and whether it attenuates biological aging. We quantified 67 ultra-marathon runners' and 56 apparently healthy males' leukocyte telomere length (T/S ratio) using real-time quantitative PCR.

The ultra-marathon runners had 11% longer telomeres (T/S ratio) than controls (ultra-marathon runners: T/S ratio = 3.5 ± 0.68, controls: T/S ratio = 3.1 ± 0.41; β = 0.40, SE = 0.10, $P = 1.4 \times 10^{-4}$) in age-adjusted analysis. The difference remained statistically significant after adjustment for cardiovascular risk factors ($P = 2.2 \times 10^{-4}$). The magnitude of this association translates into 16.2 ± 0.26 years difference in biological age and approximately 324–648bp difference in leukocyte telomere length between ultra-marathon runners and healthy controls. Neither traditional cardiovascular risk factors nor markers of inflammation/adhesion molecules explained the difference in leukocyte telomere length between ultra-marathon runners and controls. Taken together these data suggest that regular engagement in ultra-endurance aerobic exercise attenuates cellular aging.

Introduction

Regular high intense physical activity leads to an increase in cardio-respiratory fitness, which is thought to lead to subsequent reduction in risk of cardiovascular and total mortality [267-269]. Perplexingly, the anti-aging effect seems to be partly independent of traditional cardiovascular and metabolic risk factors [270,271].

Telomeres are the repeated DNA sequence located at the distal ends of linear chromosomes [272]. Without the addition of telomeric repeats by the enzyme, telomerase, somatic cell telomeres progressively shorten with each round of cell division [273]. Therefore, telomere length is a well-known indicator of mitotic replicative history and biological age. Accumulating evidence suggests that moderate amounts of physical exercise correlates with longer leukocyte telomere length [179,181,274]. Although moderate exercise has been shown as beneficial in the prevention of cardiovascular disease, chronic, excessive sustained endurance exercise such as ultra-marathon running has been reported to cause nil or even adverse effects particularly for the heart and large arteries [275]. Association studies between endurance exercise and telomere length have shown conflicting results. Previous marathon runners were found to exhibit unchanged telomere lengths in differentiated granulocytes, lymphocytes and muscle cells compared to sedentary controls [276,277]. In contrast, other studies have shown that endurance-trained athletes exhibit longer leukocyte telomeres [200,278]. Therefore, the impact of repeated, ultra-endurance aerobic exercise on telomere length and biological aging remains unclear.

An ultra-marathon is an example of extreme exposure to ultra-endurance aerobic exercise – athletes run in excess of 42 kilometers in one day. The ultra-marathon runners are an excellent model of fitness induced by repeated engagement in ultra-endurance aerobic exercise. We have previously demonstrated ultra-marathon runners have exceptionally

suppressed levels of low-grade inflammation and lower levels of low-density lipoprotein (LDL) cholesterol when compared to apparently healthy controls [279,280]. However, it is not clear whether ultra-marathon runners also benefit from attenuation of biological aging, independent of reduction in measures of cardiovascular risk. Conversely, the exposure to ultra-endurance aerobic exercise was associated with increased oxidative stress [281] that is a well-known correlate of telomere attrition rate in cellular studies [169].

Here, we measured leukocyte telomere length of male ultra-marathon runners who regularly engage in ultra-endurance running and compared them to apparently healthy controls from the general population. We also investigated whether traditional cardiovascular risk factors (including blood pressure – BP – and lipids) as well as adhesive molecules and markers of inflammation play a role in the association between ultra-endurance aerobic exercise and telomere length.

Materials and Methods

Clinical phenotyping

Sixty-seven male ultra-marathon runners and 63 age and sex-matched apparently healthy controls were included in this study. All individuals were of the same ethnicity (white Polish). The participants demographics have been outlined previously [280]. Briefly, the ultra-marathon runners had completed at least two ultra-marathons, had an average training distance of 40–100km per week and had trained for a minimum of two years [280]. All participants gave informed written consent and the study was approved by the University of Ballarat Human Research Ethics Committee. (Supplementary Method S1).

Biochemical analysis

The biochemical analyses were described before [279,280] (Supplementary Method S1).

Telomere length quantification

DNA from was extracted in the same laboratory from peripheral whole-blood by methods described elsewhere [280]. Telomere length was measured using an established quantitative real-time PCR technique [282]. This method expresses telomere length as a ratio (T/S) of telomere repeat length (T) to copy number of a single copy gene, 36B4 (S), within each sample (Supplementary Method S1).

Statistical analysis

Phenotypes with non-normal distribution underwent log-transformation before further analysis. The Student's t-test or Mann-Whitney U-test were used to examine crude differences in quantitative traits between the two groups. Linear correlation estimates were calculated using Pearson's method. Linear regression models were used to analyze telomere length in ultra-marathon runners and controls using multiple regression analyses with adjustment for age and other phenotypic covariates, and stepwise selection following adjustment for covariates. Significance was determined as $P < 0.05$. The difference in T/S ratio between ultra-marathon runners and controls was divided by the unstandardized β-coefficient from linear regression model including age and T/S ratio from a large population-based study (n > 45,000), conducted at the same laboratory using identical methodologies [283]. This provides an estimate of difference in biological age between ultra-marathon runners and controls using age-related telomere attrition rate. Others who have quantified telomere length by measuring terminal restriction fragments using the Southern Blot technique, have shown the age-related telomere attritions is approximately 20–40 base pairs (bp) per year [283]. To estimate the bp telomere length difference between cohorts, we multiplied the biological age difference (16.2 years) between ultra-marathon runners and controls by the average bp decline per year previously described – 20–40bp [283]. In doing so, we were able to estimate the

approximate difference in telomere length (expressed as bps) between ultra-marathon runners and controls.

Results

Subject Demographics

The demographic and phenotypic data are displayed in Table 1.

Ultra-marathon runners had significantly lower mean body mass index (BMI), total cholesterol (TC), soluble intracellular adhesion molecule (sICAM-1), leptin and C-reactive protein (CRP), and significantly higher mean high density lipoprotein (HDL)-cholesterol than controls.

Telomere Length and Aging

Age was inversely related to telomere length in controls (r = -0.29) and weakly – in ultra-marathon runners (r = -0.10) (Figure 1). The rate of telomere attrition (slopes of negative correlation between age and telomere length), however, was not statistically different between cohorts ($P = 0.64$) (Figure 1). The ultra-marathon runners had an 11% longer telomere length (T/S ratio) than controls (ultra-marathon runners: 3.5 ± 0.68, controls: 3.1 ± 0.41; $\beta = 0.40$, SE = 0.10, $P = 1.4 \times 10^{-4}$) in age-adjusted analysis (Figure 2).

The difference remained statistically significant after adjustment for differences between ultra-marathon runners and controls (age, BMI, TC, HDL-C, CRP, leptin, sICAM-1, PCR Plate, $\beta = 0.44$, SE = 0.14, $P = 2.2 \times 10^{-4}$) (Table 2). In the stepwise regression model (adjusting for age, interleukin-6 – IL-6, mean arterial pressure – MAP and PCR Plate) the telomere length was significantly higher in ultra-marathon runners than controls ($\beta = 0.44$, SE = 0.10, $P = 4.2 \times 10^{-5}$) (Table 2). After full adjustment, we estimated that the difference in

biological aging between ultra-marathon runners and controls was approximately 16.2 ± 0.26 years. Therefore, we estimate the ultra-marathon runners have on average approximately 324–648bp longer leukocyte telomeres compared to those of their less active peers.

Telomere Length and Cardiovascular Risk Factors

Apart from MAP, conventional cardiovascular risk factors (BMI, TC, HDL-cholesterol and triglycerides) were not associated with telomere length in ultra-marathon runners and controls (Table 3).

There was a positive correlation between MAP and telomere length in ultra-marathon runners ($r = 0.30$, $P = 0.015$). CRP, leptin, adhesion molecules (serum E-selectin – sE-selecting – and sICAM-1) and IL-6, were not significantly associated with telomere length in ultra-marathon runners, controls or joint analysis of both groups (Table 3).

Discussion

To our knowledge, this is the largest study to show that ultra-marathon runners exhibit markedly longer leukocyte telomere length compared to age-matched apparently healthy controls who do not engage in ultra-endurance aerobic exercise. We also show the impact of aging on telomere length is attenuated in ultra-marathon runners and that telomeres are approximately 16.2 years biologically younger compared to less active controls. Our results support previous data obtained from endurance-trained athletes (engaging in a similar volume of aerobic exercise) and sedentary controls [200]. However, we show that the difference in telomere length between ultra-marathon runners and controls cannot be simply explained by better cardiovascular risk profile.

Investigations on the effect of aerobic exercise on telomere length has so far provided no conclusive information on how much exercise is optimal and safe for immune cell chromosomal stability [179,181,200,260,274,276]. Sedentary middle-aged individuals exhibit shorter telomere length compared to younger and age-matched track and field athletes and endurance-trained athletes (marathon runners and triathletes) [200]. The analysis of telomere length in twin volunteers revealed the more physically active twin had longer telomeres than the less active twin [179]. Furthermore, exercise intensity is beneficial for telomere dynamics in women, as telomere length was positively associated with engaging in more frequent vigorous physical activity [284] and vigorous physical activity ameliorated telomere attrition caused by psychological stress [217]. Telomere length was also positively correlated with maximal oxygen uptake in older (55–72years) participants and it was suggested that telomere erosion was attenuated in middle-aged participants who exercise regularly [201]. In contrast, daily amount of energy expenditure had an inverted 'U'-type relationship with telomere length, in that moderate (991–2340 $Kcal \cdot wk^{-1}$) levels of energy expenditure were associated with longer telomeres compared to very low (< 991 $Kcal \cdot wk^{-1}$) and high energy expenditures (> 3541 $Kcal \cdot wk^{-1}$) [181]. We have clearly demonstrated that men who engage in ultra-endurance aerobic exercise have significantly longer telomeres compared to those who did not exercise extensively on a regular basis but otherwise were apparently healthy. Recently, marathon runners were reported to have similar lymphocyte and granulocyte telomere lengths compared to controls [276]. Potential explanations for the discrepancy between the previous findings [276] and our results may be due to the larger sample size of our study, greater age-range of participants and also due to the higher volume of aerobic exercise performed by the ultra-marathon runners included in our investigation. Interestingly, skeletal muscle telomeres are longer in endurance-trained cross-country skiers' compared to non-athletes [208]. Given the synchrony between leukocyte and skeletal muscle cell telomere shortening [285], our data along

with others' [200,208,278] support the hypothesis that both endurance and ultra-endurance exercise are beneficial to leukocyte telomere maintenance.

The longer telomeres observed in the ultra-marathon runners in our study may be a result of increased telomerase expression in leukocytes as a previous study by Werner et al. [200] showed that young and middle-aged athletes, had increased telomerase activity compared to sedentary controls [200]. Werner et al. [200] also found that athletes have differentially expressed genes associated with the shelterin complex (*TRF2*, *CHK2*, Ku 70 and 80) compared to sedentary controls [200]. Moreover, a significant increase in telomerase activity in mononuclear cells was observed after a three month intervention including 30 minutes of moderate physical activity, six days a week [214]. Recently, it was reported that following a seven day ultra-marathon footrace, ultra-marathon runners exhibited greater leukocyte mRNA content of shelterin associated genes – *TRF1, TRF2* and *POT1* [286]. The above proteins, along with several others, protect chromosomal and telomere integrity through the formation of the shelterin complex [99]. Therefore, endurance-trained individuals may benefit from ameliorated leukocyte telomere attrition by modulated shelterin and telomerase dynamics.

Our data also suggest that the difference in telomere length between ultra-marathon runners and controls cannot be simply explained by better cardiovascular risk profile in those who engage in regular ultra-endurance aerobic exercise. Indeed, neither traditional cardiovascular risk factors nor markers of inflammation/adhesion molecules showed association with telomere length, and their inclusion in the regression model had no effect on the association between telomere length and ultra-endurance aerobic exercise. Although there was no significant difference in the MAP between the ultra-marathon runners and controls we observed a positive correlation between leukocyte telomere length and MAP in ultra-marathon runners but not the controls. The biological mechanisms of this somewhat paradoxical correlation are not clear. Interestingly, previous findings have shown that

telomere length is positively related to left ventricular mass [287], that in turn is a direct associate of blood pressure. In this context the correlation seen in our study may be explained (at least in part) by the adaptation to chronic endurance exercise. On the other hand, we should acknowledge that blood pressure is a rapidly changing physiological parameter and the value of single clinic measurements may not necessarily reflect the long-term effect of BP on cardiovascular system, in particular when taken in a relatively small group of individuals. Larger population samples are necessary to fully dissect the association between BP and telomere length in ultra-marathon runners.

We should, however, acknowledge that several unmeasured intermediate phenotypes may be relevant here. Although not measured directly, cardiorespiratory fitness gained from previous extensive training would be significantly better in the ultra-marathon runners than controls.

Maximal oxygen uptake has been positively correlated with telomere length in older, endurance-trained adults [201]. Interestingly, patients with longer telomeres and greater exercise capacity had reduced mortality risk [207]. Therefore, it is tempting to postulate that increasing amounts of ultra-endurance aerobic exercise may be beneficial to decreasing mortality risk through cardiovascular training adaptations, potentially leading to an extended lifespan.

In the current study we found that biologically ultra-marathon runners are approximately 16.2 years younger than less physically active controls, equating to an approximate 324–648bp longer telomeres than controls. Notably, endurance-trained athletes' (> 55years) telomeres, measured by Southern Blot, were shown to have approximately 900bps longer leukocyte telomeres than sedentary individuals [278]. Engaging in greater amounts of physical activity has been shown previously to have anti-aging effects. Ultra-endurance athletes have 17% greater longevity compared to the general population [288], and numerous studies have demonstrated decreased mortality with more frequent exercise [269,289]. With telomere length a marker of

biological age, less active individuals exhibit 10 years biologically older leukocytes compared to their more active peers [179]. Healthy individuals have 11 years biologically younger leukocytes compared to patients with CVD [290]. Moreover, coronary artery disease patients with greater exercise capacity exhibited longer telomeres compared to patients with a lower exercise capacity, representing a four year difference in biological age [207]. In this context, a 16 year difference in biological age between ultra-marathon runners and controls appears particularly significant and its implications for health and disease needs to be further elucidated.

Our study has a number of limitations. Information on diet [291] and psychological stress [292] which have been demonstrated to influence telomere dynamics were not recorded in our participants. Our study was cross-sectional in nature and therefore we were unable to assign direct causative nature to the association between telomere attrition and physical exercise. Future studies should investigate telomere erosion longitudinally, measuring telomeres at multiple time points in people engaging in different physical activity levels, to gain a better insight into the protective effect physical exercise may have on cellular aging. Moreover, delineation of the molecular pathways modulated by exercise, which are responsible for telomere maintenance, is of high priority.

In conclusion, our results are the first to demonstrate that chronic ultra-endurance aerobic exercise is associated with slower cellular aging by attenuated telomere length attrition, independent of age and traditional markers of cardiovascular risk, as well as markers of inflammation/adhesion molecules. They also demonstrate that ultra-endurance exercise does not have adverse effects on the cardiovascular system through telomere attrition.

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Figure legends

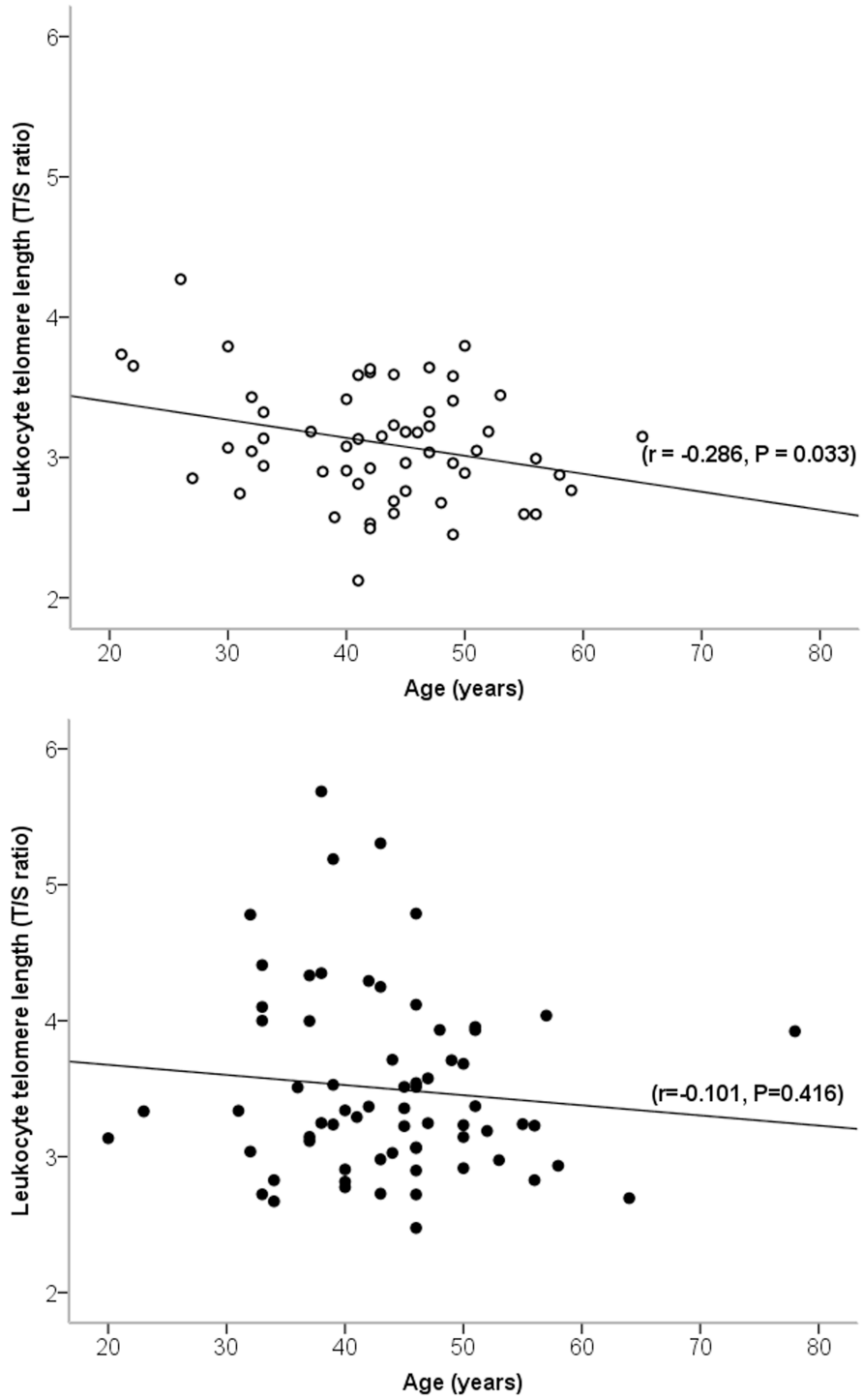

Figure 1. Pearson's linear correlation between age and leukocyte telomere length in ultra-marathon runners and controls. Ultra-marathon runners are indicated by filled circles and controls are indicated by empty circles.

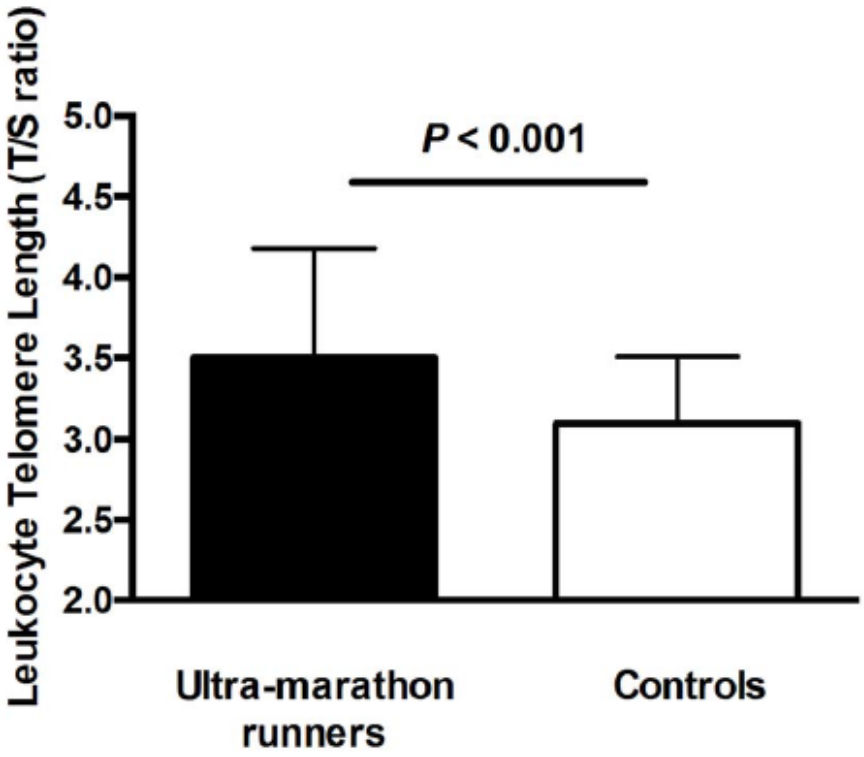

Figure 2. Telomere length comparison between ultra-marathon runners and controls. Mean leukocyte telomere length is presented in arbitrary units as the telomere to single copy gene (T/S) ratio. Error bars represent standard deviation.

Table 1. Clinical phenotypes of ultra-marathon runners and apparently healthy controls.

	Ultra-marathon runners (n = 67)	**Controls (n = 56)**	***P*-value**
Age (years)	43.6 ± 9.2	42.8 ± 9.2	0.62
BMI (kg/m^2)	23.2 ± 2.0	25.2 ± 2.5	$\mathbf{2.7 \times 10^{-6}}$
MAP	95.8 ± 5.4	95.9 ± 8.2	0.92
TC (mmol/L)	5.1 ±1.0	5.7 ± 1.0	**0.0014**
HDL-C (mmol/L)	1.2 ± 0.3	1.0 ± 0.3	$\mathbf{6.7 \times 10^{-4}}$
Triglycerides (mmol/L) *	1.7 (1.37, 1.86)	1.6 (1.37, 1.86)	0.70
CRP (mg/L) *	0.4 (0.34, 0.59)	1.4 (1.05, 1.91)	$\mathbf{2.6 \times 10^{-8}}$
IL-6 (pg/mL) *	1.3(1.11, 1.46)	1.5 (1.26, 1.74)	0.10
Leptin (ng/mL) *	2.1 (1.76, 2.40)	5.6 (4.34, 7.20)	$\mathbf{3.6 \times 10^{-9}}$
sE-selectin (ng/mL) *	49.8 (44.51, 55.76)	46.1 (40.67, 52.18)	0.46
sICAM -1 (ng/mL) *	202.1 (186.53, 219.06)	232.8 (210.38, 257.56)	**0.015**

Data are from either Student's t-test or Mann-Whitney U-tests and are expressed as means and standard deviations or geometric means and 95% confidence intervals (*); BMI – body mass index, MAP – mean arterial pressure, TC – total cholesterol, HDL – high-density

lipoprotein cholesterol, CRP – C-reactive protein, IL-6 – interleukin-6, sICAM-1 – soluble intercellular.

Table 2. Difference in leukocyte telomere length between ultra-marathon runners and controls.

Model	**Covariates adjusted for**	**β-coefficient (95%CI)**	***P*-value**
Basic	Age	0.40 (0.103)	**1.4×10^{-4}**
Fully adjusted model 1 (stepwise)	Age, IL-6, MAP and PCR Plate	0.44 (0.103)	**4.2×10^{-5}**
Fully adjusted model 2 (forced)	Age, BMI, TC, HDL-C, CRP, leptin, sICAM-1, PCR Plate	0.44 (0.140)	**2.2×10^{-4}**

The differences are expressed as unstandardized β-coefficients with standard errors from either stepwise linear regression (Fully adjusted model 1) or linear regression (Fully adjusted model 2 – Forced), MAP – mean arterial pressure, IL-6 – interleukin-6, PCR Plate – experiment used in measurement in LTL, BMI – body mass index, TC – total cholesterol, HDL-C – high-density lipoprotein cholesterol, CRP – C-reactive protein, sICAM – soluble intercellular adhesion molecule-1.

Table 3. Linear correlation between leukocyte telomere length and cardiovascular health markers, adhesion molecules, cytokines and inflammation markers.

	All		Ultra-marathon runners		Controls	
	r	*P*-value	r	*P*-value	r	*P*-value
Cardiovascular risk factors						
BMI (kg/m^2)	-0.13	0.15	0.08	0.50	-0.13	0.33
TC (mmol/L)	-0.04	0.69	0.05	0.69	0.14	0.33
HDL-C (mmol/L)	0.03	0.76	-0.11	0.37	-0.04	0.75
MAP	0.13	0.16	**0.30**	**0.015**	-0.03	0.80
Triglycerides (mmol/L)	0.02	0.85	0.10	0.42	0.13	0.35
Inflammation/adhesion molecules						
CRP (mg/L)	-0.09	0.29	0.11	0.37	-0.03	0.84
IL-6 (pg/mL)	-0.12	0.20	-0.10	0.44	-0.06	0.68
Leptin (ng/mL)	-0.15	0.09	0.006	0.96	0.07	0.62
sE-selectin (ng/mL)	0.02	0.85	0.01	0.92	-0.06	0.67
sICAM -1 (ng/mL)	-0.14	0.11	-0.05	0.69	-0.16	0.25

BMI – body mass index, TC – total cholesterol, HDL-C – high-density lipoprotein cholesterol, MAP – mean arterial pressure, CRP – C-reactive protein, IL-6 – interleukin-6,

sE-selectin – serum E-selecin, sICAM-1 – Soluble intercellular adhesion molecule-1. Data from Pearson's Correlations are expressed by r and p-values.

Chapter 4 Increased expression of telomere-regulating genes in athletes with long leukocyte telomeres – published in *Journal of Applied Physiology, 2016*

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INCREASED EXPRESSION OF TELOMERE-REGULATING GENES IN ENDURANCE ATHLETES WITH LONG LEUKOCYTE TELOMERES

Running head: Telomere-regulating genes in endurance athletes

Joshua Denham[1,4], Brendan J. O'Brien[2], Priscilla R. Prestes[3], Nicholas J. Brown[2], Fadi J. Charchar[3,4,*]

[1] School of Science and Technology, University of New England, Armidale, NSW, Australia.

[2] Faculty of Health, Federation University Australia, Mount Helen, Victoria, Australia.

[3] Faculty of Science and Technology, Federation University Australia, Mount Helen, Victoria, Australia.

[4] Australian Centre for Research into Injury in Sport and Its Prevention (ACRISP), Federation University Australia, Mount Helen, Victoria, Australia.

*Corresponding author: Prof Fadi Charchar, Y Building, University Drive, Federation University Australia, Mt Helen, Victoria, Australia 3350. Ph: +613 5327 6098, Fax: +613 5327 9602, Email: f.charchar@federation.edu.au

Keywords: Resting heart rate, shelterin, *TERT*, VO_{2max}, sitting

Abstract

Leukocyte telomeres shorten with age and excessive shortening is associated with age-related cardio-metabolic diseases. Exercise training may prevent disease through telomere length maintenance though the optimal amount of exercise that attenuates telomere attrition is unknown. Furthermore, the underlying molecular mechanisms responsible for the enhanced telomere maintenance observed in endurance athletes is poorly understood.

We quantified the leukocyte telomere length and analysed the expression of telomere-regulating genes in endurance athletes and healthy controls (both n = 61), using quantitative PCR.

We found endurance athletes have significantly longer (7.1%, 208–416 nt) leukocyte telomeres and up-regulated *TERT* (2.0-fold) and *TPP1* (1.3-fold) mRNA expression compared to controls in age-adjusted analysis. The telomere length and telomere-regulating gene expression differences were no longer statistically significant after adjustment for resting heart rate and relative $\dot{V}O_{2max}$ (all $p > 0.05$). Resting heart rate emerged as an independent predictor of leukocyte telomere length, *TERT* and *TPP1* mRNA expression in stepwise regression models. To gauge whether volume of exercise was associated with leukocyte telomere length, we divided subjects into running and cycling tertiles (distance covered per week) and found individuals in the middle and highest tertiles had longer telomeres than individuals in the lowest tertile. These data emphasise the importance of

cardiorespiratory fitness and exercise training in the prevention of biological aging. They also support the concept that moderate amounts of exercise training protects against biological ageing, while higher amounts may not elicit additional benefits.

Introduction

Telomeres are repetitive DNA (in mammals, 5'-TTAGGG-3') positioned at the ends of chromosomes that protect against genomic DNA degradation and chromosomal fusion events [7,8]. Due to the end replication problem, telomeres shorten in the absence of telomerase with each round of cell-division and as such telomere length is an established marker of ageing [293-295]. Telomeres and six telomere-regulating proteins (telomere repeat-binding factor 1 [TRF1], telomere repeat-binding factor 2 [TRF2], TRF1-interacting nuclear factor 2 [TINF2], adrenocortical dysplasia homolog [TPP1], protection of telomeres 1 [POT1] and TRF2-interacting protein [TERF2IP]), collectively called shelterin, form nucleoprotein complexes that maintain genomic stability and regulate telomere length. Shelterin is crucial for telomerase-mediated telomere length maintenance and genomic stability, as removal of shelterin causes severe telomere and chromosomal aberrations [63,99,296]. Telomerase is comprised of telomerase reverse transcriptase (TERT) and the telomerase RNA component (*TERC*), and can combat premature ageing by extending telomeric DNA [9,297].

Telomere length of proliferative tissues, such as leukocytes, is longest at birth and shortening is dependent on genetic and lifestyle factors. Psychological stress [72], poor diet [126] and age-related diseases including coronary artery disease [70], obesity [132] and diabetes [15] are all associated with excessive leukocyte telomere shortening. Conversely, mounting evidence has unveiled a positive influence of physical activity levels on leukocyte telomere length [179-181,200,201,217,298]. Lifestyle interventions including increases in moderate-intensity physical activity extends telomere length after a five-year period [215]. Although exercise seems to

benefit telomere length, the ideal amount of exercise training for telomere length maintenance and the underlying molecular mechanisms remain elusive.

We previously reported that relative to healthy controls, ultra-marathon runners had, on average, 11% longer leukocyte telomeres, indicating that they had prevented ~16 years' worth of age-related telomere attrition [202]. German National Track and Field athletes have increased TRF2 protein content and up-regulated telomerase activity in peripheral blood mononuclear cells (PBMC) compared to sedentary controls [200]. Furthermore, PBMC shelterin gene (TRF1, TRF2 and POT1) expression was up-regulated after a seven day ultra-marathon event [231]. Thus, shelterin and other telomere-regulating genes may underpin the longer leukocyte telomeres associated with long-term endurance exercise training. A comprehensive analysis of all shelterin and *TERT* gene expression between endurance athletes and healthy controls has not yet been performed.

Subsequently, the purpose of our study was to extend previous findings by determining whether any association between telomere length and exercise was mediated through telomere-regulating gene expression in endurance athletes. A further aim was to establish whether linear associations exist between physical activity, cardiorespiratory fitness and leukocyte telomere length.

Materials and Methods

Participants

A total of 122 Caucasian subjects were recruited from the general public and participated in this study. Subjects were deemed apparently healthy – non-smoking, not taking any medications and free from any age-related chronic diseases – according to self-reported health questionnaires. Endurance athletes (n = 61) and recreationally active controls (n = 61), aged 18 to 55 y were analysed. Endurance- athletes trained were cyclists, triathletes, middle-

or long-distance runners and ultra-marathon runners at state through to international level. Endurance athletes trained >3 times per week and had trained consistently for a minimum of one year. The apparently healthy controls were recreationally active but were not engaged in any structured aerobic or resistance exercise training.

All participants gave written informed consent and this study was approved by Federation University Australia's Human Research Ethics Committee.

Procedures

Subjects physical activity levels and psychological stress was assessed by the self-administered International Physical Activity Questionnaire (IPAQ) Long form [299] and Perceived Stress Scale (PSS) [300], respectively. Data cleaning and analysis was performed according to the IPAQ guidelines and average weekly Metabolic Equivalent of task (MET) – minutes and sitting were calculated and included as continuous variables in statistical analyses. Height, weight and body mass index (BMI) were recorded and subjects were seated for approximately 10 minutes before BP assessment. The SphygmoCor device (AtCor Medical, Australia) was used to assess brachial blood pressure, averaged from three separate measurements, taken one minute apart with subjects seated. Subjects' cardiorespiratory fitness, determined as maximal oxygen consumption ($\dot{V}O_{2max}$), was assessed through a maximal graded treadmill or cycle ergometer test via pulmonary analysis. While control subjects completed a maximal treadmill test, the endurance cyclist completed a cycle ergometer test. Triathletes obtain a comparable $\dot{V}O_{2max}$ value regardless of exercise mode [301] and as such, triathletes from the present study completed either a cycle or treadmill test. Before maximal exercise testing subjects were fitted with a two-way breathing valve (Hans Rudolph) and expired air was collected into an online metabolic system (Moxus, Modular,

USA) for O_2 and CO_2 analysis. The metabolic system was calibrated prior to each test using ambient air and gas of known composition. The treadmill commenced at 10 $km \cdot h^{-1}$ and was progressively increased by 1 $km \cdot h^{-1}$ every two minutes until volitional exhaustion. Cycle ergometer $\dot{V}O_{2max}$ tests commenced at 100 W and the load was increased by 30 $W \cdot min^{-1}$ every two minutes until pedalling cadence dropped below 50 RPM for 10 seconds or until volitional exhaustion. Subjects were asked to maintain 90–100 RPM throughout cycle ergometer-assessed exercise tests. Individual $\dot{V}O_{2max}$ was determined as the highest O_2 value averaged over 60 seconds.

Telomere length quantification

A preprandial blood sample (~20 ml) was drawn from the antecubital vein into EDTA tubes using standard phlebotomy procedures. All subjects gave a seated resting blood sample 24 to 48 hours after their last exercise session. DNA was extracted from whole-blood leukocytes using the Purelink Genomic DNA Mini Kit (Life Technologies, Australia). Telomere length was quantified using an established qPCR method [13,14,202,302], previously validated by terminal restriction fragment analysis [13]. Within each sample, the telomere repeat copy number (T) is compared to a single copy gene copy number (S) and expressed in arbitrary units as a (T/S) ratio. Briefly, 10µl reactions comprised of 2 × SensiFast SYBER Lo-ROX master mix (Bioline, Australia), primer sets and 10ng of DNA, were run in triplicate on the ViiA7 Real Time PCR System (Life Technologies, Australia). Either 300nM of telomere-specific forward (5'GGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT3') and reverse (5'GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT3') primers, or 300nM of forward (5'CAGCAAGTGGGAAGGTGTAATCC3') and 500nM of reverse (5'CCCATTCTATCATCAACGGGTACAA3') primers for the 36B4 gene was used in

reactions. All samples were run with a positive and no template controls on a single 384-well plate to prevent any inter-plate variability. The cycling conditions telomere assays was as follows: a hold at 95° for 10 min, followed by 40 cycles at 95° for 15 s and 58° for 1 min. As a quality control, samples were excluded from the analysis if the difference between triplicates was greater than one cycle threshold (Ct), or the average of duplicates was taken for further analysis. The intra-assay coefficient of variation between triplicate samples was 2.5% and 1.4% for the telomere and 36B4 gene, respectively.

Gene expression analysis

Leukocytes were isolated as previously described [248] and RNA was extracted using the miRVana miRNA Isolation Kit (Life Technologies, Australia), following the manufacturer's guidelines. RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit (Life Technologies). Telomere-regulating gene expression was quantified using SYBR or TaqMan chemistries. Primer-sets and TaqMan Assays (Life Technologies) are outlined in Table 1. An efficiency curve was generated for each primer-set using cDNA diluted 1:2 from 50ng to 3.125ng. The qPCR product was run on an agarose gel to ensure appropriate amplicon length and a single product. Triplicate samples were run on a single 384-well plate with negative controls. The cycling conditions for primer-based assays was: a hold at 95° for 2 min, followed by 40 cycles at 95° for 5 s, 60° for 10 s and 72° for 20 s. Cycling for TaqMan assays was: a hold at 50° for 2 min and another at 95° for 20 sec, followed by 40 cycles at 95° for 1 s and 60° for 20 s. Relative gene expression was assessed using the $2^{-\Delta\Delta Ct}$ method [303]. Whilst differential gene expression between athletes and controls was represented by fold-difference, gene expression analysis involving all subjects was represented using relative gene expression compared to the control mRNA, *GAPDH*. The

coefficient of variation between triplicates for each of the mRNAs ranged from 0.66 to 1.49% (Table 1).

Statistical analysis

Using data from our previous cross-sectional study [202], our *a priori* power analysis revealed we required a sample size of 88 (44 in each group) in order to achieve >90% power to detect a difference ($d > 0.7$) in leukocyte telomere length between athletes and controls (G*Power, version 3.1.5). All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 21, IBM Corp, NY). Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Two-way independent samples *t*-tests or Mann-Whitney U-tests were used to examine differences in physical characteristics and fitness parameters, and telomere length between athletes and controls. To control for covariates, an ANCOVA was used to establish differences between athlete and control telomere length and telomere-regulating gene expression. An ANOVA was also used to determine telomere length differences between subjects divided into cycling and running distance tertiles. Spearman's correlations were used on to examine associations between physical characteristics and fitness parameters, with telomere length and telomere-regulating gene expression. Stepwise linear regression was performed to identify predictors of telomere length and telomere-regulating gene expression. Statistical significance was set at $p < 0.05$. The difference in biological age and telomere length – expressed as nucleotides (nt) – between athletes and controls was estimated using the same calculations as described previously [202].

Results

Physical characteristics

The controls were five years younger than the athletes ($p = 0.06$). Relative to the controls, the athletes had a lower body weight, resting heart rate and had a higher cardiorespiratory fitness as indicated by their $\dot{V}O_{2max}$ and maximal treadmill speed (all $p < 0.001$, Table 2). Athletes engaged in less sitting and were more physically active compared to their non-athletic peers (all $p < 0.01$, Table 2).

Linear correlations between telomere length, age, health and exercise phenotypes

Age was not statistically correlated to telomere length in all subjects or when athletes and controls were analysed separately (all $p > 0.05$, Table 3). When athletes and controls were pooled we found weak to moderate correlations between telomere length and weight, BMI, systolic blood pressure and resting heart rate (n = 122, all $p < 0.05$, Figure 1). Furthermore, we found correlations between cardiorespiratory fitness and physical activity parameters – Metabolic equivalent of task-min per week, time spent sitting and maximal treadmill speed – and leukocyte telomere length (all $p < 0.05$, Figure 2). In athletes, years spent training was not associated with telomere length (n = 60, r = -0.12, $p = 0.37$).

Telomere length analysis

Relative to the controls, the endurance athletes had 7.1% longer leukocyte telomeres after age-adjustment (T/S ratio ± SE: 3.64 ± 0.06 v 3.38 ± 0.06, $p = 0.002$, Figure 3A). The biological age difference between endurance athletes and controls translated to 10.4 years, meaning the athletes had prevented 10.4 years of biological ageing. We estimated the

biological age difference was equivalent to the athletes possessing 208–416 nt longer telomeres compared to the controls. Compared to controls, athletes had lower body weight and resting heart rate, and a higher cardiorespiratory fitness (Table 2, all $p < 0.001$). To determine whether these phenotypes mediated the leukocyte telomere length difference found between athletes and controls, we performed an additional analysis including these phenotypes as covariates. After adjusting for age, weight, resting heart rate and relative $\dot{V}O_{2max}$, however, the difference between athletes and controls was no longer statistically significant (T/S ratio ± SE: 3.58 ± 0.08 vs 3.45 ± 0.08, $p = 0.36$).

We then performed a stepwise linear regression to determine predictors of leukocyte telomere length. After including health and fitness parameters – age, height, weight, body mass index, systolic, diastolic, mean arterial and pulse pressure, and relative $\dot{V}O_{2max}$ – in the stepwise regression model, resting heart rate emerged as the only independent predictor of leukocyte telomere length amongst athletes and controls, such that it explained 10.1% of the overall variation (B = -0.012, CI: 3.85–4.625, $p < 0.001$).

Telomere-regulating gene expression analysis

Relative to controls, endurance athletes had 2.0-fold and 1.3-fold up-regulated *TERT* (Figure 3B) and *TPP1* (Figure 3C) mRNA expression, respectively. No other telomere-regulating genes – *TERC*, *TERF2IP*, *TINF2*, *TERF1*, *TERF2* and *POT1* – were differentially regulated between athletes and controls ($p > 0.05$, Table 4). The up-regulated *TERT* and *TPP1* mRNA expression remained statistically significant after adjusting for health phenotypes ($p = 0.005$ and $p = 0.05$, respectively). After further adjustment for heart rate and relative $\dot{V}O_{2max}$, however, the difference was no longer statistically significant ($p = 0.16$ and $p = 0.41$). Besides *TERC* (r = -0.28, $p = 0.003$), there were no other statistically significant correlations

between telomere length and expression of any of the telomere-regulating genes analysed ($p > 0.05$, Table 5). *TERT* and *TPP1* were both correlated with resting heart rate and relative $\dot{V}O_{2max}$ (Figure 3D–G).

Again, we performed stepwise regression including health and fitness parameters and found that resting heart rate was an independent predictor of *TERT* mRNA expression, explaining 9.4% of the variation (Table 6). Age, height and resting heart rate were independent predictors of *TPP1* mRNA expression, together explaining 9.5% of the variation (Table 6).

Moderate amounts of exercise training associated with long telomeres and increased TERT and TPP1 mRNA expression

To establish associations between volume of exercise training and telomere length, we divided subjects into tertiles for weekly running and cycling distance and analysed telomere length. We found that age-adjusted telomere length was significantly longer in subjects in the middle and highest tertiles for weekly running and cycling distance (Figure 4A and B, respectively) compared to those in the lowest tertile. A similar relationship was observed between weekly training distances and *TERT* and *TPP1* mRNA expression (Figure 4D, E, G and H). Moreover, individuals with the highest cardiorespiratory fitness had longer leukocyte telomeres, up-regulated *TERT* and *TPP1* mRNA expression compared to those in the lowest tertile with poor cardiorespiratory fitness (Figure 4C, F and I, respectively). No statistically significant differences were found between those in the middle and highest cardiorespiratory fitness tertiles for telomere length, *TERT* and *TPP1* mRNA expression.

Lower resting heart rate is associated with longer telomeres

To investigate the association between resting heart rate and telomere length we divided our subjects into resting heart rate tertiles and found a linear decrease in leukocyte telomere length with a higher resting heart rate (Figure 4J). Subjects with a resting heart rate below 50 beats·min^{-1}, on average, exhibited 14.4% and 8.5% longer telomeres compared to those with a resting heart rate 51–74 and >75 beats·min^{-1}, respectively (Figure 4J). A similar relationship was also observed between resting heart rate and *TERT* and *TPP1* mRNA expression (Figure 4K and L, respectively).

Discussion

Endurance athletes who regularly engage in high volumes of exercise training have preserved leukocyte telomeres [200-202] though the underlying molecular and physiological determinants remain incompletely understood. Here, we not only verified that endurance athletes have significantly longer leukocyte telomeres, but we also wanted to determine if the longer telomeres observed in athletes was caused by the modulation of gene expression in telomere length regulating genes. We found that the adrenocortical dysplasia homolog (TPP1) and TERT genes were both up-regulated in leukocytes from athletes compared to controls. The longer leukocyte telomeres and increased *TERT* and *TPP1* mRNA expression observed in endurance athletes appears to be associated with their lower resting heart rate and superior $\dot{V}O_{2max}$.

The majority of previous research has shown physical activity is positively correlated to leukocyte telomere length [179-181,200,201,217], though the optimal amount for telomere length maintenance remains unclear. For instance, some researchers suggest moderate amounts of physical activity is ideal for telomere maintenance [181,182], whilst studies on endurance athletes – who regularly engage in strenuous endurance exercise training – supports the premise that

higher volumes of endurance exercise is conducive to telomere protection [200-202]. Here, we verify previous studies [201,202] indicating endurance athletes possess significantly longer leukocyte telomeres (by 7.1%, 208–416 nt) compared to controls of average cardiorespiratory fitness. Our previous investigation on ultra-marathon runners revealed they had 324–648 nt longer telomeres, which translated to 16.2 years less telomere attrition compared to healthy controls [202]. The endurance athletes in the present study were, on average, five years older than the controls yet possessed longer leukocyte telomeres to a relatively similar magnitude as found in our previous study [202]. The average telomere length difference between endurance athletes and controls from the present study indicated the endurance athletes possessed telomeres as long as controls 10.4 years their junior, providing additional evidence that endurance exercise training attenuates biological ageing.

Although previous studies [200-202] and our findings indicate endurance exercise training is associated with longer telomeres, the molecular mechanisms leading to longer leukocyte telomeres in endurance athletes is unclear. Up-regulation of telomerase is a likely mechanism of longer telomeres in athletes. German track and field and endurance athletes accumulating an average of >70 km of running per week, exhibited up-regulated peripheral blood mononuclear *TRF2* mRNA and protein expression, with increased telomerase activity [200]. Here, we found increased whole-blood leukocyte *TERT* and *TPP1* mRNA expression in endurance athletes. It is possible that repeat bouts of exercise training may reprogram *TERT* and *TPP1* mRNA expression, which would improve telomerase activity and processivity, and ultimately preserve telomere length. Previous analyses involving mononuclear cells [200], *TRF2* mRNA was not differentially expressed in our endurance athletes, potentially due to the different cell type studied – whole blood leukocytes. Increased mononuclear cell *TRF1*, *TRF2* and *POT1* mRNA expression was observed in endurance athletes the day after a 183-mile ultra-marathon race [231], but these shelterin genes were not differentially expressed in our

athletes in a rested state. TERT is the major protein component of the reverse transcriptase, *telomerase* [304], with a known role in preventing replication-induced telomere shortening [108,305]. Interestingly, leukocyte *TERT* mRNA expression was increased (19.4-fold) after a 30-min run at 80% of $\dot{V}O_{2max}$ in healthy men [230]. Therefore, considering POT1 together with TPP1 help recruit and increase the repeat processivity of telomerase [93], the increased *TERT* and *TPP1* mRNA expression found in athletes from our study and up-regulated leukocyte telomerase activity in athletes' from others [200] may contribute to the underlying molecular mechanisms by which endurance exercise training preserves leukocyte telomeres. Pathways activated by aerobic exercise training, such as the nitric oxide synthase, Akt protein kinase, insulin growth factor-1 signalling [200,228] and p38 mitogen-activated protein kinase [232] are candidate signalling cascades that may regulate telomerase activity-dependent telomere maintenance via *TERT* activation.

Interestingly, age was not negatively correlated to leukocyte telomeres in athletes, control or pooled subjects. This may be due to the narrow age range (18–55 y) or alternatively because the controls were recreationally active. Body weight, body mass index, systolic BP, mean arterial pressure and resting heart rate were all inversely correlated to leukocyte telomere length. Consistent with previous studies [201,206-208], we found a positive correlation between cardiorespiratory fitness, assessed by $\dot{V}O_{2max}$ testing, and telomere length. Interestingly, *TERC* mRNA expression was inversely correlated to telomere length. Potential explanations for this finding is that elevated *TERC* mRNA expression may not be required in the absence of excessive telomere shortening, experimental noise or because *TERC* is not the rate limiting factor for telomerase activity. Providing evidence that longer leukocyte telomeres are reflective of physical performance capabilities and physical activity, we found maximal treadmill speed and physical activity were positively correlated to leukocyte telomere length. A recent randomised, controlled trial revealed reduced time spent sitting was associated with

telomere lengthening in a group of sedentary older adult (68 y) men and women [190]. We found time spent sitting per week was inversely correlated to leukocyte telomere length in younger (~30 y) subjects. Notably, the athletes in the present study reported sitting much less relative to controls (4.8 v 10.8 $hr \cdot day^{-1}$). It may be that the longer leukocyte telomeres possessed by endurance athletes is result of both extensive exercise training and less sedentary time (i.e. more physical activity). Therefore, these data suggest increased physical activity, cardiorespiratory fitness and limited time spent sitting contribute to telomere maintenance, in turn, protecting against cardiovascular disease and biological ageing.

We also found *TERT* and *TPP1* mRNA expression were positively and inversely correlated to $\dot{V}O_{2max}$ and resting heart rate, respectively. To our knowledge we are the first to show such a relationship between parameters of cardiorespiratory fitness – $\dot{V}O_{2max}$ and resting heart rate – and telomere-regulating gene expression. An increase in $\dot{V}O_{2max}$ and lowering of resting heart rate are adaptations to endurance exercise training [306,307]. Interestingly, the differences in leukocyte telomere length, *TERT* and *TPP1* mRNA expression between athletes and controls was no longer statistically significant after adjustment for $\dot{V}O_{2max}$ and resting heart rate, indicating these parameters may be important for telomere length maintenance.

Exceptional arterial health and cardiac capacity (primarily stroke volume) are required for a high $\dot{V}O_{2max}$ and maybe the underlying biological mechanisms explaining the observed association with telomere length maintenance. The shorter leukocyte telomeres observed in patients with atherosclerosis is well known [13,14,70,308] and shortening of leukocyte telomeres is more pronounced in individuals with atherosclerotic progression over a six [178] and ten [177] year time period. Leukocyte telomere length reflects the telomere length of haematopoietic stem cells [309], which are precursors for endothelial progenitor cells [310]. Subsequently, endurance exercise training may attenuate telomere shortening in haematopoietic stem cells

and, in turn, conserve the replicative potential of endothelial progenitor cells to ultimately conserve arterial health and function.

The stepwise inverse association between lower resting heart rate and leukocyte telomere length has multiple explanations. For example, exercise-training induced bradycardia involves decreased sympathetic nervous system activation and increased peripheral arterial compliance [306]. Increased oxidative stress production in medulla of rats leads to sympathetic activation and hypertension [311]. Telomeres are particularly vulnerable to shortening caused by inflammation [168] and oxidative stress [83,169], and both are implicated in cardiovascular disease [312-314]. Endurance athletes, however, have low circulating markers of inflammation [280] and exercise training leads to up-regulated antioxidant enzyme activity [315,316]. Therefore, whilst speculative, ameliorated inflammation and oxidative stress, with up-regulate telomere-associated genes caused by endurance exercise training may protect against telomere shortening, but this requires additional investigation.

Most studies have found a positive relationship between the amount of physical activity and leukocyte telomere length, but the optimal amount of exercise for telomere preservation is not known. Another novel aspect of our study was that after dividing subjects into tertiles for running and cycling distance covered per week, we found individuals in the middle and highest tertiles for exercise training possessed similar leukocyte telomere lengths that were longer compared to those in the lowest exercise tertile. A similar relationship was observed with *TERT* and *TPP1* mRNA expression, suggesting that exercise-induced benefits to telomere length maintenance maybe conferred by moderate and high amounts of exercise training. The practical application of these findings are that individuals who wish to maintain their leukocyte telomere length could benefit from running more than 10 km a week, but running more than 25 km a week may not provide additional telomere preservation. Similarly, cycling greater than 200 km a week may be unnecessary for telomere length

maintenance, rather a minimum of 30 km cycling a week could elicit attenuate age-related telomere attrition. These data are somewhat supported by findings from epidemiological studies on physical activity measured in context with cardiovascular disease and mortality risk. A meta-analysis indicated the risk of coronary heart disease is reduced by 14% and 20% in individuals engaging in the recommended 150 and 300 minutes, respectively, of moderate-intensity physical activity per week [3]. The relative risk of coronary heart disease, however, was only modestly lower in those engaging in the highest amount – 750 minutes – of physical activity per week [3]. In a cohort of 55,137 adults, the relative risk reduction in all-cause and cardiovascular mortality was reduced in runners compared to non-runners but the decreased risk was achieved with as little as running ~10 km per week [317]. We found a linear relationship between leukocyte telomere length and resting heart rate. Resting heart rate has long been recognised an independent risk factor for cardiovascular disease and all-cause mortality, with higher resting heart rates eliciting a greater risk [318-320]. Leukocyte telomere length is also a predictor of cardiovascular disease [13,152] and all-cause mortality [22,321]. Therefore, it is possible that aerobic exercise training-induced telomere maintenance could occur in conjunction with lowering of resting heart rate and this, in turn, may ameliorate the risk of cardiovascular disease and mortality. This study was not designed to investigate the possible causal role exercise-induced lowering of resting heart rate has on leukocyte telomere length and disease and mortality risk. Future research should establish how improvement to cardiorespiratory fitness and a reduction of resting heart rate maintains telomere length.

We had over 90% power to detect a difference in leukocyte telomeres, which is a strength of the study. Whilst we acknowledge our data does not directly show that endurance exercise training maintains leukocyte telomere length, the alternative explanation would be that being born with long telomeres might be associated with a markedly higher cardiorespiratory performance and instinctive willingness to engage in extensive exercise training; an

alternative and plausible explanation. A limitation of the study is that dietary analysis was not performed therefore we cannot account for the potential impact of diet on leukocyte telomere biology. Leukocyte protein was not collected therefore future studies should confirm the *TERT* and *TPP1* mRNA expression differences amongst athletes and controls at the translational level. Given that critically short telomeres promote cellular senescence [322], it would be advantageous to study the percentage of short telomeres in context with physical activity and cardiorespiratory fitness, rather than mean telomere length outlined in the present study. Although our statistical analysis indicated key cardiorespiratory fitness adaptations – lower resting heart rate and superior $\dot{V}O_{2max}$ – partly explained the telomere length difference found between athletes and controls, additional studies are required to delineate the physiological mechanism. Our data was correlative and does not infer causation. Future work should focus on the molecular mechanisms regulating telomere length dynamics in context with exercise training. It will be important to determine the genetic contribution of long telomeres from the influence of exercise training. Considering $\dot{V}O_{2max}$ and resting heart rate are heritable traits, accounting for ~50% [222] and 13 to 60% [323-325] of the variation, respectively, it could be that endurance athletes from our study inherited long telomeres and their involvement in exercise training is coincidental. Longitudinal analyses are required to appreciate whether and what type of exercise training, and underlying physiological adaptations, attenuates the rate of telomere shortening in humans, to prevent biological ageing and disease.

In summary, endurance athletes possess longer leukocyte telomeres and up-regulated *TERT* and *TPP1* mRNA expression. Our findings indicate a role for $\dot{V}O_{2max}$ and lower resting heart rate in the benefits that endurance exercise training has on leukocyte telomere maintenance. We also found a plateauing effect between the amount of running and cycling distance covered per week and increasing leukocyte telomere length. Therefore, this suggests that

moderate amounts of exercise (running: 10 to 25 km·week^{-1}; cycling: 30 to 200 km·week^{-1}) may be as sufficient as large amounts of exercise to prevent age-associated telomere erosion.

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Disclosures

The authors have no conflicts of interest to disclose.

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Tables

Table 1. Primer-sets and assay identification numbers.

Gene symbol	Primer-sets/Assay ID	CV (%)
TERT	F: GAA GAA GCC ACC TCT TTG GA	1.36
	R: AGA GAG CTG AGT AGG AAG GAG	
POT1	F: GCT CTG GCT TTG CAT CTT TG	0.82
	R: GGT GCC ATC CCA TAC CTT TAG	
TINF2	F: CAA GTC CTG AAA GCC CTG AA	1.32
	R: CTT TCT CCA GCT GAC ACA AGT A	
TPP1	F: CCA CGC TGC TTG TGT CT	1.05
	R: GCG GTC CAC CTG GAG ATA	
TERF1	F: ACC CTT GAT GCA CAG TTT GA	1.49
	R: CTG CCT TCA TTA GAA AGG TTG ATG	
TERF2	F: CAC ACC ACT GGA ATC AGC TAT C	0.66
	R: CAG GAT GGG CCA AGT TCT TT	
GAPDH (control)	F: GGG TGT GAA CCA TGA GAA GT	0.98
	R: AGT AGA GGC AGG GAT GAT GT	
TERF2IP	Hs00430292_m1	0.71
TERC	Hs03454202_s1	1.30
GAPDH (control)	Hs02786624_g1	1.03

Legend: ID, identification number (Life Technologies); CV, coefficient of variation (intra-plate).

Table 2. Characteristics of endurance athletes and controls.

Variable	Endurance athletes (n = 61)	Controls (n = 61)	*p*-value
Men/women (n)	46/15	47/14	
Age (y)	33.7 ± 11.03	28.7 ± 10.64	0.06
Ht (cm)	176.36 ± 10.10	173.82 ± 8.97	0.14
Wt (kg)	70.56 ± 10.69	78.65 ± 10.96	**< 0.001**
BMI (Wt/Ht^2)	22.6 ± 2.23	26.02 ± 2.95	**< 0.001**
SBP (mm Hg)	124.96 ± 10.91	125.75 ± 10.65	0.68
DBP (mm Hg)	73.44 ± 8.08	75.95 ± 9.11	0.11
PP (mm Hg)	51.52 ± 7.97	49.46 ± 9.45	0.20
MAP* (mm Hg)	90.46 ± 8.3	92.44 ± 8.73	0.20
Resting HR ($beats \cdot min^{-1}$)	51.62 ± 7.58	68.67 ± 10.62	**< 0.001**
$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	58.77 ± 8.75	43.73 ± 7.03	**< 0.001**
Maximum treadmill speed ($km \cdot h^{-1}$)	17.02 ± 1.97	13.23 ± 1.92	**< 0.001**
Maximum wattage (w)	370.23 ± 69.38	-	**-**
PSS	12.21 ± 4.81	11.36 ± 5.74	0.39
Sitting ($min \cdot wk^{-1}$)	2010 (1290–2700)	4560 (2220–8460)	**< 0.001**
EEE ($Mj \cdot wk^{-1}$)	32.43 (23.23–55.7)	23.64 (8.92–40.65)	**0.002**
METs ($min \cdot wk^{-1}$)	6976 (4878–13116)	3528 (1556.5–7520.5)	**< 0.001**
Years trained (y)	5.5 (2.62–12)	2.25 (0–8.5)	**< 0.001**
Run distance ($km \cdot wk^{-1}$)	40 (30–60)	2.5 (0–10)	**< 0.001**
Cycle distance ($km \cdot wk^{-1}$)	150 (0–237.5)	-	**-**

Swim distance ($km \cdot wk^{-1}$)	4.5 (0–8)	-	-

Data are expressed as mean ± standard deviation or median (interquartile range) from two-tailed independent samples *t*-tests or Mann-Whitney U-tests.

Legend: Ht, Height; Wt, Weight; BMI, body mass index; SBP, systolic BP; DBP, diastolic BP; PP, pulse pressure (SBP-DBP); MAP, mean arterial pressure *calculated by ((2×diastolic)+systolic)÷3; HR, heart rate; $\dot{V}O_{2max}$, maximal aerobic (cardiorespiratory) fitness; PSS, perceived stress scale; EEE, estimated energy expenditure; METs, metabolic equivalent of task.

Table 3. Linear correlations between age and telomere length in athletes and controls.

	All subjects (n = 122)		Athletes (n = 61)		Controls (n = 61)	
Variable	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
Age	0.03	0.74	0.04	0.78	-0.12	0.35

Data are from Spearman's correlations.

Table 4. Telomere-regulating gene expression in athletes and controls ($p > 0.05$).

Gene	**FD**
TERC	1.27
TRF1	0.91
TRF2	0.93
TINF2	0.90
POT1	0.97
TERF2IP	1.03

Data are expressed as fold-difference relative to controls (FD = 1).

Table 5. Linear correlations between telomere length and telomere-associated gene expression.

	All subjects (n = 121)	
Gene	**r**	***p*-value**
TERT	0.09	0.315
TERC	**-0.28**	**0.003**
TRF1	-0.08	0.35
TRF2	0.05	0.55
TPP1	0.12	0.25
TINF2	0.07	0.48
POT1	-0.007	0.93
TERF2IP	0.05	0.61

Data are from two-tailed Spearman's Correlation.

Table 6. Stepwise regression models for *TERT* and *TPP1* mRNA expression.

Dependent variable	**Predictors**	**Unstandardised B-value**	**SE**	***t*-value**	***p*-value**	$r^2_{(adj)}$
TERT	HR	-1.24	0.34	-3.64	< 0.001	0.094
TPP1	Age	0.33	0.16	2.01	0.047	0.095
	Height	0.45	0.18	2.15	0.01	
	HR	-0.26	0.14	-1.89	0.06	

Data are from stepwise linear regression. Variables excluded from the models for *TERT* include: age, height, weight, body mass index, systolic, diastolic, pulse and mean arterial pressure, and $\dot{V}O_{2max}$. Variables excluded from the models for *TPP1* include: weight, body mass index, systolic, diastolic, pulse and mean arterial pressure, and $\dot{V}O_{2max}$.

Legend: SE, standard error; HR, resting heart rate.

Figure legends

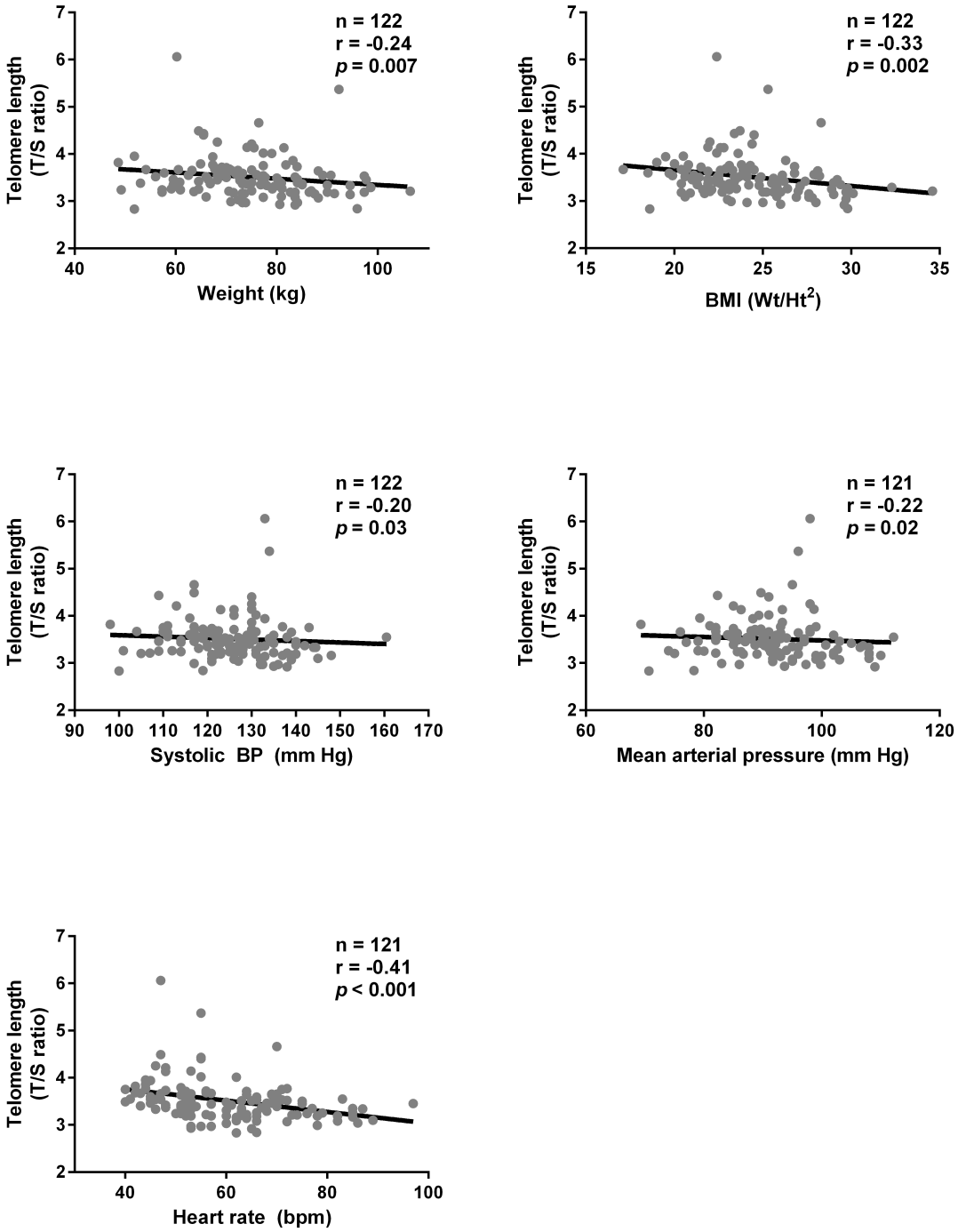

Figure 1. Linear correlations between leukocyte telomere length and health parameters.

Data are from Spearman's correlations.

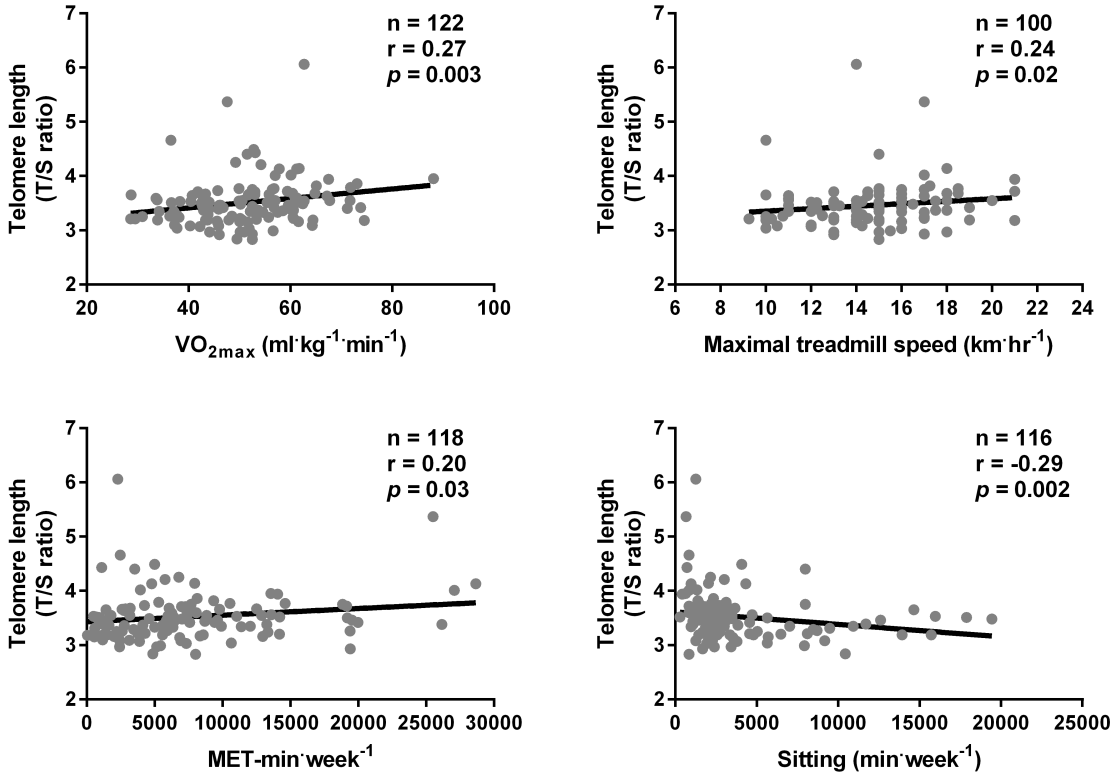

Figure 2. Linear correlations between leukocyte telomere length and exercise parameters. Data are from Spearman's correlations.

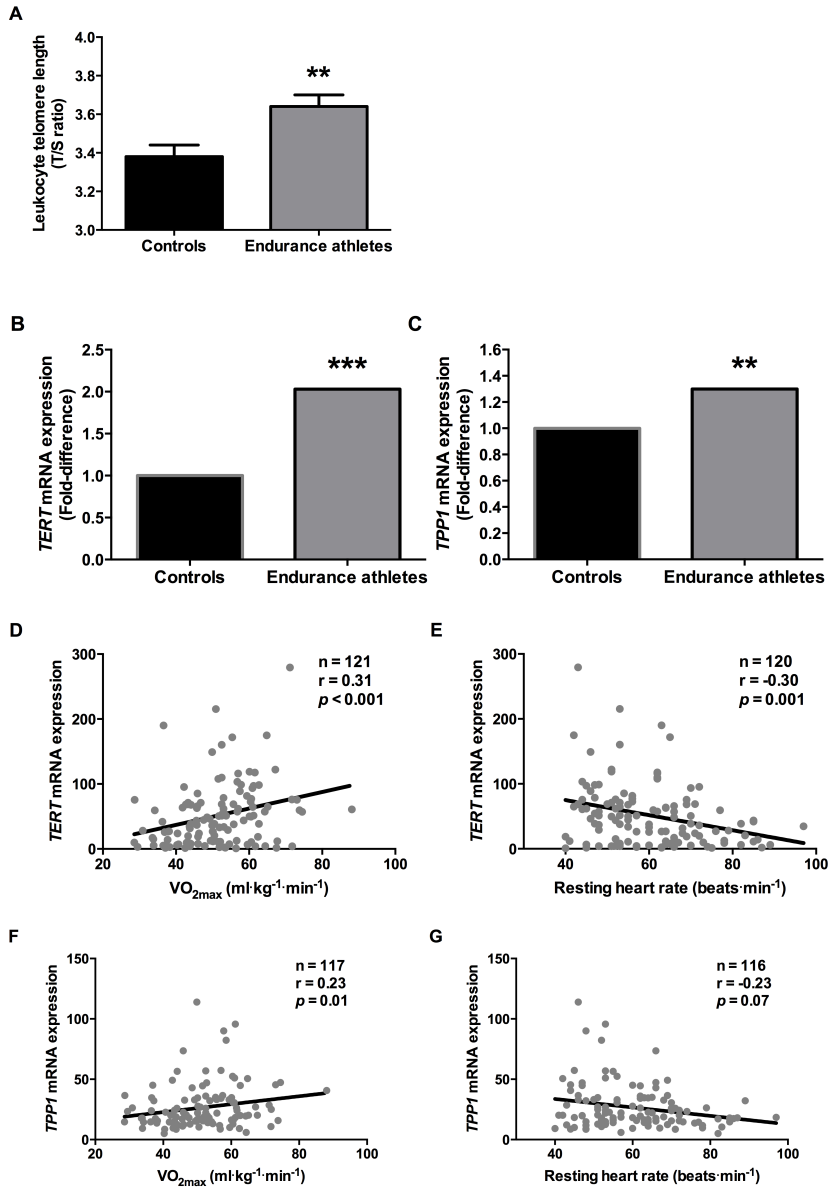

Figure 3. Endurance exercise, telomere length, and *TERT* and *TPP1* mRNA expression.

A) Leukocyte telomere lengths adjusted for age are from an ANCOVA including 61 athletes and controls. Bars and whiskers indicate mean and standard error, respectively. Relative to controls, endurance athlete had increased *TERT* (B) and *TPP1* (C) mRNA expression (athletes vs controls [relative expression ± SE]: 68.31 ± 7.03 vs 34.07 ± 4.3, $p < 0.001$ and 31.39 ± 2.93 vs 21.53 ± 1.56, $p = 0.004$, respectively). Data are from Mann-Whitney U test. Correlations between *TERT* mRNA expression, $\dot{V}O_{2max}$ (D) and resting heart rate (E).

Correlations between *TPP1* mRNA expression, $\dot{V}O_{2max}$ (F) and resting heart rate (G). Data are from Spearman's correlations. $^{**}p < 0.01$; $^{***}p < 0.001$.

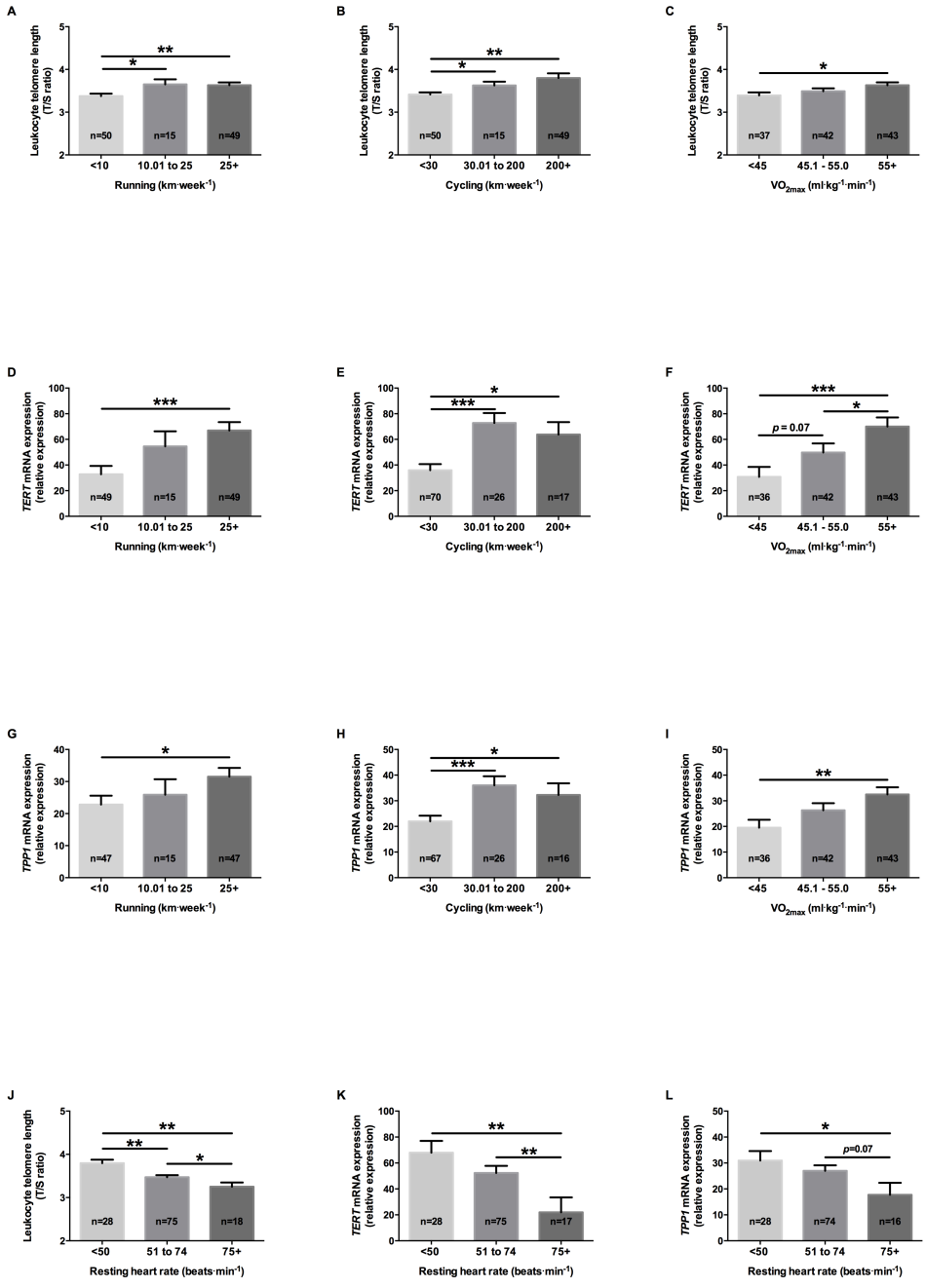

Figure 4. Moderate amounts of exercise training and lower resting hear rates are associated with longer leukocyte telomeres. Telomere length was analysed in context with running (A) and cycling (B) distance, and $\dot{V}O_{2max}$ tertiles (C). Similarly, *TERT* (D, E and F) and *TPP1* (G, H and I) mRNA expression was analysed in context with running, cycling and

$\dot{V}O_{2max}$, respectively. Heart rate tertiles were formed and analysed in context with telomere length (J), *TERT* (K) and *TPP1* (L) mRNA expression. Bars and whiskers indicate mean±SE from an ANCOVA, adjusted for age. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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Exercise – putting action into our epigenome

Short title: Exercise and epigenetics

Joshua Denham, Francine Z. Marques, Brendan J. O'Brien, Fadi J. Charchar

School of Health Sciences, University of Ballarat, Ballarat, VIC, Australia

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Corresponding Author

Fadi Charchar, Room 228, F Building, Oppy Drive, University of Ballarat, Mt Helen, 3350.

P: (03) 5327 6098, Fax: (03) 5327 9602

Email: f.charchar@ballarat.edu.au

Figure Captions

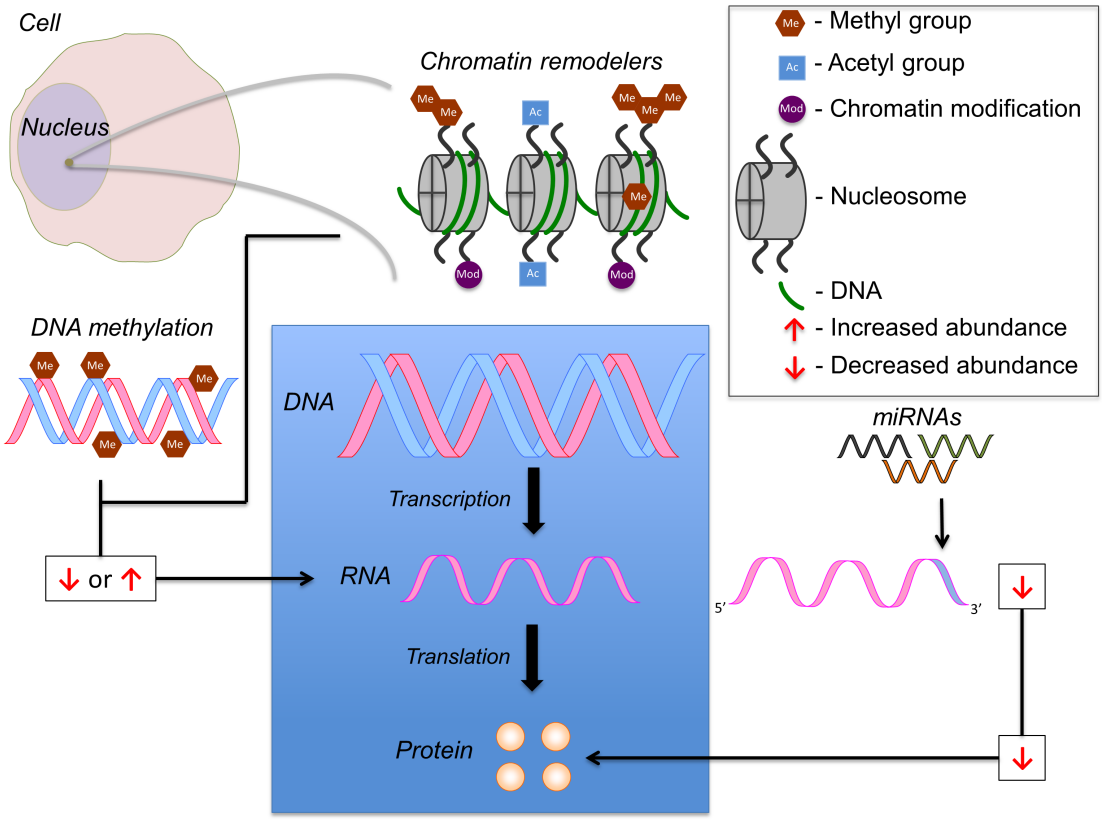

Figure 1. Impact of epigenetic modifications and microRNAs (microRNAs). DNA is wrapped around an octomer complex, known as a nucleosome, made up of four pairs of histone proteins, plus histone H1 (linker protein). Histone protein sites (eg. lysines and arginines) are vulnerable to epigenetic modifications, such as acetylation, methylation and several other chemical modifications; of which change the formation of chromatin to an open, transcriptionally active or a closed, transcriptionally repressed state. DNA methylation, however directly changes the structure of DNA and in doing so allows active transcription or silencing of genes, depending on the location. Although not working above the genome, miRNAs function by mostly targeting specific 3' untranslated regions (UTR) of messenger RNA (mRNAs) and negatively regulate protein abundance by post-transcriptional regulation of mRNA stability or through the degradation of mRNA molecules. The binding of 5'UTRs is also possible, leading to either up- or down-regulation of mRNA levels (not shown in figure).

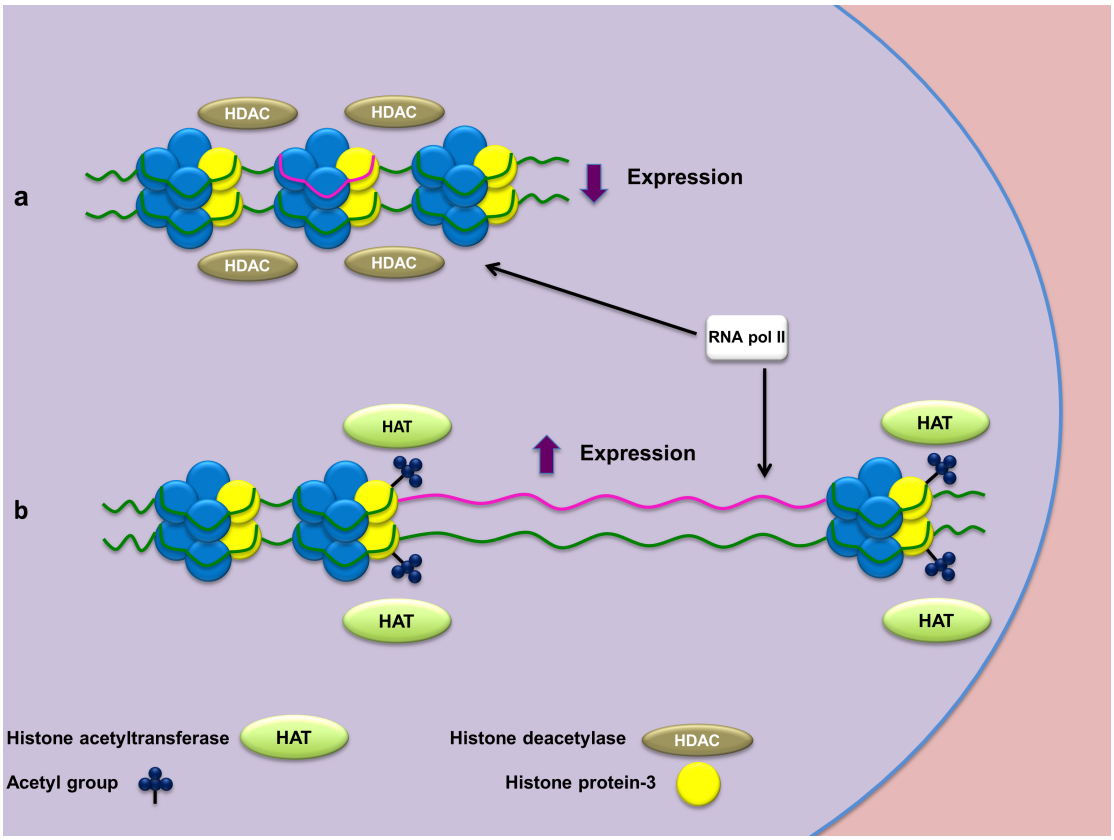

Figure 2. Mechanisms of action of histone acetylation. (a) A gene (indicated by pink lines) is elegantly stored around three nucleosomes. The histone-3 proteins (yellow circles) are deacetylated at lysine-9 (H3K9) by histone deacetylase (HDAC) enzymes – an epigenetic mark of transcriptional repression. (b) Two nucleosomes housing a gene have been acetylated at histone-3 lysine-9 (H3K9ac) – an epigenetic mark of transcriptional activation, favouring gene transcription. This epigenetic modification is carried out by histone acetyltransferase (HAT) enzymes.

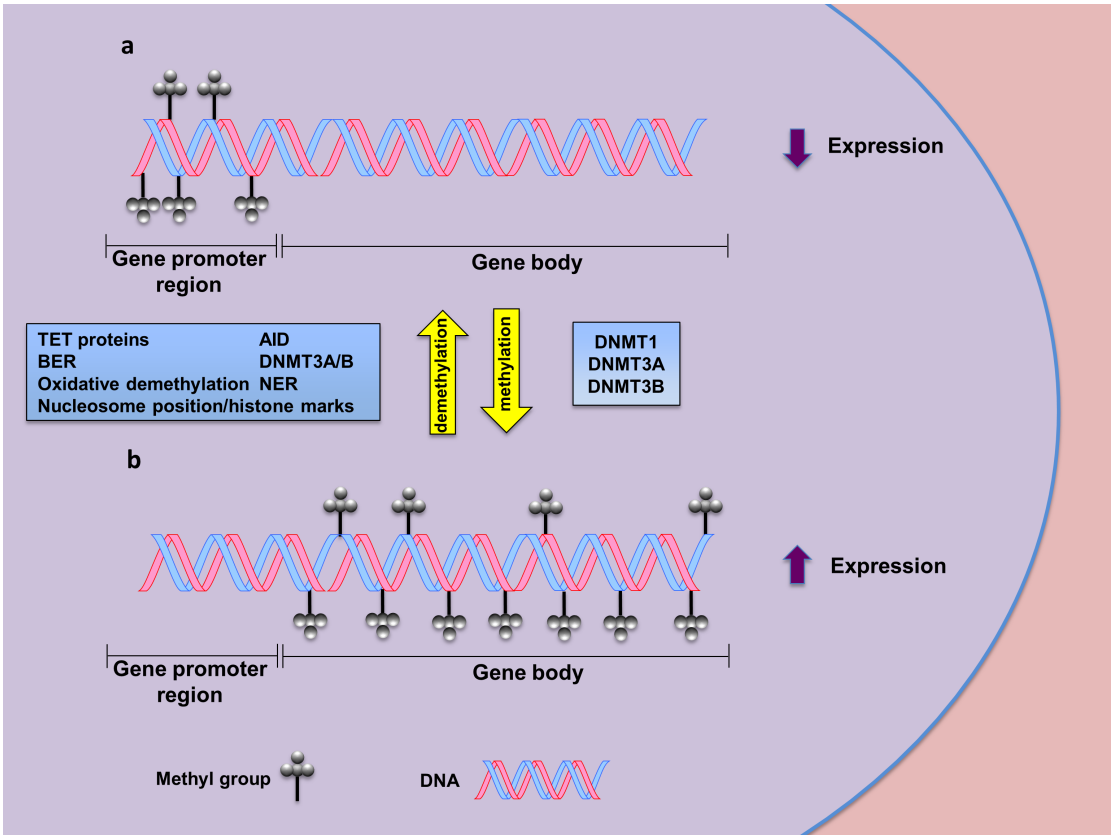

Figure 3. The effect DNA methylation has on gene expression is dependent on its location within the genome. (a) DNA methylation within a gene's promoter region is associated with transcriptional repression. (b) Conversely, DNA methylation within the gene body is associated with transcriptional active DNA. DNA methylation is actively performed predominantly by DNA methyltransferase-1 (DNMT1), through the transfer of an accepted methyl group from a donating S-adenosylmethionine (SAM). DNMT3A and 3B, however, are vital for embryonic DNA methylation and early development. Active and passive DNA demethylation is potentially conducted through many mechanisms (for detailed review the readers are referred elsewhere [326,327]).

DNMT1 = DNA methyltransferase-1; DNMT3A = DNA methyltransferase-3A; DNMT3B = DNA methyltransferase-3B; TET = Ten-eleven translocation protein (1-7); AID = activation-induced cytidine deaminase; BER = Base excision repair; NER = Nucleotide excision repair.

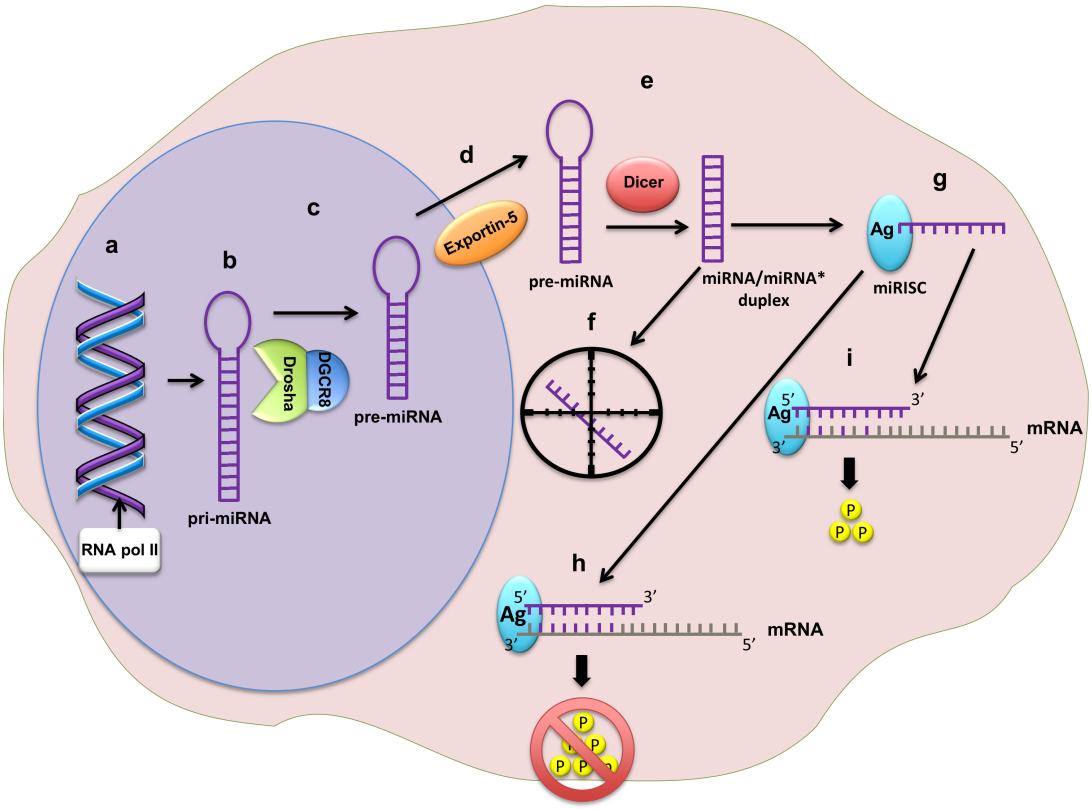

Figure 4. microRNA (miRNA) biogenesis and down-regulation by binding to the 3' untranslated region (UTR) of a messenger RNA (mRNA). (a) A gene coding a miRNA is transcribed by RNA polymerase II (RNA pol II), (b) producing a primary-miRNA (pri-miRNA). (c) The pri-miRNA is cleaved by the multi-protein complex, Drosha/Di George Syndrome critical region gene 8 (DGCR8), creating a preliminary-miRNA (pre-miRNA). (d) The pre-miRNA is transported from the cell nucleus to the cytosol by Exportin-5, (e) where it is further cleaved by Dicer, transforming it into the miRNA/passenger miRNA (miRNA/miRNA*) duplex. (f) The passenger miRNA (miRNA*) can be degraded or recently it has been suggested to have a role in mRNA regulation too. (g) Any one of several argonaute proteins (Ag) attaches to the mature miRNA, thereby creating the miRNA-induced silencing complex (miRISC) assembly. (h) The miRISC is able to either regulate protein (P) abundance by negative repression of transcribed RNA when the seeding region of the miRNA is partially complementary to the 3'UTR of the target mRNA (~7 bases in length), or

(i) it can lead to mRNA repression and subsequent degradation when the seeding region is completely complementary to the 3'UTR of the target mRNA.

Abstract

Most human phenotypes are influenced by a combination of genomic and environmental factors. Engaging in regular physical exercise prevents many chronic diseases, decreases mortality risk and increases longevity. The mechanisms involved, however, are poorly understood. The modulating effect of physical (aerobic and resistance) exercise on gene expression has been known for some time now and has provided us with an understanding of the biological responses to physical exercise. Emerging research data suggest that epigenetic modifications are extremely important for both development and disease in humans. In the current review, we summarise findings on the effect of exercise on epigenetic modifications and their effects on gene expression. Current research data suggests epigenetic modifications (DNA methylation and histone aceytlation) and microRNAs (miRNAs) are responsive to acute aerobic and resistance exercise in brain, blood, skeletal and cardiac muscle, adipose tissue and even buccal cells. Six months of aerobic exercise alters whole-genome DNA methylation in skeletal muscel and adipose tissue and directly influences lipogenesis. Some miRNAs are related to maximal oxygen consumption (VO_{2max}) and VO_{2max} trainability, and are differentially expressed amongst individuals with high and low VO_{2max}. Remarkably, miRNA expression profiles discriminate between low and high responders to resistance exercise (miR-378, -26a, -29a and -451) and correlate to gains in lean body mass (miR-378). The emerging field of exercise epigenomics is expected to prosper and additional studies may elucidate the clinical relevance of miRNAs and epigenetic modifications, and delineate mechanisms by which exercise confers a healthier phenotype and improves performance.

Introduction

The human phenotype is influenced directly, or in combination with, our genes and the environment. There is a genetic predisposition to many diseases.[328-331] Interestingly, little is known as to how environmental stimuli impact our phenotype and what molecular mechanisms are involved

Epigenetics is the study of changes to gene expression, independently of genotype that, in certain instances, are trans-generationally heritable. The epigenome consists of a plethora of DNA and chromatin modifications that influence gene expression by performing conformational changes inside the nucleus of cells and governs tissue specific gene expression.[332,333] Aberrant epigenetic profiles are associated with numerous diseases.[55,334,335] Environmental stimuli may induce epigenetic modifications, which in turn, modulate gene expression. Furthermore, microRNAs (miRNAs) impact protein abundance through post-transcriptional regulation of messenger RNA (mRNA) and the expression of numerous miRNAs become dysfunctional with disease.[336,337]

Physical exercise is a financially viable and modifiable lifestyle choice underutilised by many. The health and fitness benefits conferred by regular aerobic and resistance exercise training include alleviated risk of developing and reduced severity of some cardiovascular, metabolic and pulmonary diseases, obesity and certain types of cancer.[56,338,339]

In this review, we discuss data showing the influence that exercise has on DNA methylation, histone acetylation and miRNAs, and their impacts on gene expression and phenotype.

Biology of epigenetic modifications and microRNAs

The interaction of genetic and environmental factors significantly influences human phenotypes, including the susceptibility to many common diseases. Despite the original idea the human genome is inert and imperturbable to structural changes, epigenetic studies have revealed quite the contrary.[340] The epigenome consists of a plethora of potent gene regulatory mechanisms (histone acetylation, DNA methylation, etc; see Table I for definitions of key terms).[341,342] Translated, 'epi' means above and 'genome' describes the heritable blueprints of an organism – the DNA. Therefore, these epigenetic modifications function by rearranging the conformation of DNA or the architecture of structures holding DNA (nucleosomes) inside the cell nucleus (Figure 1). In doing so, they act to encourage or prevent transcription, through an intricate interplay of molecular pathways to date not completely understood.[332]

Although numerous epigenetic modifications are known,[342] only DNA methylation and histone acetylation have been investigated in context with physical exercise. These include histone acetylation and DNA methylation, discussed below.[343-346] A detailed examination and outline of the intricate pathways involved in signalling epigenetic modifications is outside the scope of this review; these have been outlined previously.[332,347] Moreover there is great diversity in genetic assays for quantifying miRNA and epigenetic modifications, and these have been described elsewhere.[348-352]

Histone Acetylation

Nucleosomes consist of four, paired histone molecules (H2A, H2B, H3 and H4) and H1 (linker protein) of which approximately 150 base-pairs (bp) of DNA is wrapped around and stored. Epigenetic modifications at histone proteins alters chromatin structure and modulates gene expression, and these are dependent on the location and type of epigenetic modification (acetylation, phosphorylation, deamination, ubiquitylation, sumoylation, etc.), or degree of histone methylation (mono-, di- or tri-methylation) (Figure 1).[342] Not all chromatin

modifications have been profiled in response to exercise and therefore this review will focus on those previously analysed with exercise, specifically, histone acetylation and DNA methylation. Histone proteins acquire an acetyl group (acetylation) through mechanisms reliant on the enzyme, histone acetyltransferases (HATs); conversely deacetylation is conducted predominantly by deacetylase enzymes (HDACs) (1-11).[353] Acetylation of histone proteins shifts nucleosome position and in doing so, either promotes or represses gene transcription by exposing sites necessary for the binding of the transcriptional machinery (Figure 2).[332]

DNA Methylation

Another widely studied epigenetic modification is DNA methylation. DNA methylation involves the addition of a methyl group to the 5th carbon of a cytosine neighbouring a guanine nucleobase (CpG – p indicates the phosphate ion between the nucleobases).[354] The human genome is predominantly methylated at CpG sites,[355,356] yet un-methylated CpG 'islands' are interspersed in repetitive groups throughout the genome and are most frequently found at the promoter region of genes.[356] Although not as common as CpG sites, non-CpG DNA methylation is possible and may have regulatory roles in cellular functions and tumour pathogenesis.[357]

The effect DNA methylation has on gene expression is dependent on its location within the genome. DNA methylation at the promoter and enhancer regions of genes is associated with transcriptional repression whereas the un-methylated state is related to a transcriptionally permissive state.[356] Conversely, DNA methylation within the gene body is associated with active transcription (Figure 3).[358] DNA methylation changes the conformational layout of chromatin to a more condensed state, inaccessible to the transcriptional machinery. Moreover, methyl-binding proteins mediate histone modifications

and direct DNA methylation cross-talk with histone modifications and also contribute to the DNA methylation related transcriptional silencing.[359,360]

DNA methylation, in conjunction with other epigenetic modifications is vital for genomic imprinting, as well as the transcriptional silencing of a single female X-chromosome.[361-363] Moreover, the increased DNA methylation at the promoter regions of tumour suppressor genes and loss of long interspersed repeat sequences (LINE-1) DNA methylation is associated with the pathogenesis of cancer.[364-367] Interestingly, discordant DNA methylation profiles are exhibited between monozygotic twins, who have phenotype discordances and who are otherwise genetically identical.[340,368,369] Importantly, epigenetic landscapes are cell type specific and contribute to the unique gene expression profile.[370]

DNA methylation is actively regulated by the enzyme family, DNA methyltransferases (DNMT), consisting of DNMT1, DNMT3A, DNMT3B and DNMT3L (Figure 3).[371] Whereas DNMT1 is primarily responsible for replicating DNA methylation in dividing cells[371] and also for chromosomal stability,[372] DNMT3A and 3B are required for establishing early embryonic development and corresponding DNA methylation patterns.[373] DNMT3L, however, lacks the catalytic activity to methylate CpG sites but catalyses DNMT3A- and DNMT3B-induced DNA methylation.[374] DNA methylation is conducted by DNMT1 through the acquisition of a methyl group donated by the methyl-donor, S-adenosylmethionine.[375] How CpG sites are demethylated is not completely understood, but a number of possible mechanisms have been hypothesised. These include, the conversion of carbon 5-methylcytosine to 5-hydroxmethylcytosine by the ten-eleven translocation (TET) proteins 1-3,[376] base excision repair (BER) of 5-methylcytosine, deamination of 5-methylcytosine followed by BER, oxidative DNA demethylation and activation-induced deaminase (AID) and these have been discussed in recent reviews.[326,327]

microRNAs

miRNAs are non-coding RNAs of approximately 18-24 bases long and regulate mRNA and/or protein abundance by binding to either the 3' untranslated region (UTR) of bases 2-7 (seed region) or 5' UTR of mRNAs.[349,377] The binding of miRNAs to a 3'UTR leads to the decrease in the stability of the mRNA, resulting in its degradation, or to the repression of the translation of the mRNA into protein.[13] More recently, the binding of miRNAs to the 5' UTR of mRNAs has been found to either up or down-regulate translation.[377,378] Although some recent investigations showed that miRNAs are also able to up-regulate gene expression, there are no such examples in the literature related to exercise, and therefore, this will not be discussed in this review.

The miRNA biogenesis is illustrated in Figure 4. Briefly, RNA polymerase II transcribes a miRNA-coding region, commonly found within genes,[379] but are also located across the genome,[380,381] thereby producing a primary miRNA (pri-miRNA). The enzyme, Drosha, along with the protein, Di George Syndrome critical region gene 8 (DGCR8), aid the formation of a preliminary miRNA (pre-miRNA) by cleaving the pri-miRNA.[382,383] Once transported from the cell nucleus to the cytosol by Exportin-5,[384] the pre-miRNA is cleaved a second time by Dicer, consequently making the miRNA/passenger miRNA duplex.[385,386] The mature, functional miRNA is finally added to the RNA-induced silencing complex (RISC) together with an argonaute protein and is now able to regulate translation.[387,388]

Similarly, to epigenetic modifications, miRNA expression is cell type-specific.[389] These miRNAs have enormous potential to regulate gene expression, as a single miRNA can have several mRNA targets. They are estimated to target more than 30% of the genes in the human genome and over 1,000 miRNAs are identified.[390-392] Dysfunctional miRNA expression are observed in patients with cardiovascular disease, cancer and type 2 diabetes mellitus –[393-395]

these are diseases that are also partially prevented and attenuated by regular physical exercise.[56]

Potential mRNA targets are predicted by matching their nucleotide sequence to that of a complementary miRNA sequence. Conveniently, databases are available to search for predicted mRNA targets for specific miRNAs using advanced algorithms, such as TargetScan, miRanda, PicTar and microRNA.org. Furthermore, other useful websites and software for miRNA assays and nomenclature include miRBase and MiRConverter, respectively. Notably, it is imperative to validate 'predicted' miRNA target gene transcripts, using luciferase assays or other *in vitro* experiments; this ensures justified conclusions are made regarding the biological function of the miRNA/s under investigation. Furthermore, there are numerous technical considerations, such as RNA extraction, platform and house-keeping miRNA selection, when conducting miRNA studies and these have been extensively discussed by others.[396-398]

Therefore, epigenetic modifications and miRNAs have the capacity to significantly influence transcription and translation, which may result in physiological and subsequent phenotypic changes. Epigenetic modifications work through an intricate molecular network to either encourage or repress gene expression by performing conformation changes to chromatin. Similarly, post-transcriptional gene regulation by miRNAs adds further complexities to the heavily governed expression of genes. Most intriguingly, both epigenetic modifications and miRNAs are associated with human health and disease, and are influenced by physical exercise.

The influence of exercise on epigenetic modifications

Modifiable lifestyle and environmental factors such as diet and exercise significantly influence gene expression, and this is in part orchestrated by epigenetic modifications (see Table II for summary of physical exercise-induced epigenetic responses). Here, we review the current literature on the influence that exercise has on histone acetylation and DNA methylation in different cell types.

Brain

Aerobic physical exercise produces numerous health benefits in the brain. Regular engagement in physical exercise elicits enhanced cognitive functioning, increased brain neurotropic proteins, such as brain derived neurotrophic factor (BDNF), and prevents cognitive diseases.[399-401] Recent findings highlight a role for aerobic exercise in modulating chromatin remodelers.[346,402-405] Histone-3 (H3) phosphorylation at serine-10 (Ser10) along with acetylation at lysine-14 (K14ac) was significantly increased in brain tissue (hippocampi) of rats, 15 minutes following the completion of 10 minutes of swimming exercise. [346] Histone H3 lysine 14 acetylation (H3K14ac) peaked two hours after swimming exercise, returned to a non-significant amount 24 hours after exercise had ceased. It was mediated by pathways involving the N-methyl-D-aspartate receptor, extracellular signal-regulated kinases 1/2, mitogen and stress related kinase 1 and 2.[346] These results were the first to demonstrate acute and relatively short aerobic exercise modulates epigenetic modifications. The transient epigenetic modifications observed due to chronic running training have also been associated with improved learning and stress-coping strategies, epigenetic changes and increased c-Fos positive neurons in dentate gyrus of rats.[403]

BDNF is a protein involved in neurogenesis, brain development and learning,[406] and exercise promotes BDNF production.[407] One week of voluntary running caused concurrent increased *Bdnf* mRNA (41%) and protein (30%) abundance with significant DNA de-

methylation of the *Bdnf* exon 4 promoter region (CpG site, 148 bp downstream) in rat hippocampi.[408] Using chromatin immunoprecipitation (ChIP) assays, the authors revealed exercised rats exhibited marked H3 acetylation (H3ac) but not H4ac, possibly as a result of decreased *Hdac5* mRNA (25%) and protein (91%) abundance, and pathways involving cAMP-dependent, catalytic, α-protein kinase (cAMP), response element binding protein (Creb) and Ca^{2+}/calmodulin-dependent protein kinase (Camk) phosphorylation.[408] Histone acetyltransferase and deacetylase enzymes were up- and down-regulated, respectively, following acute but not chronic wheel running.[409] In contrast to previous findings,[408] exercise induced increase in HAT activity was found only on H4.[409] Others have demonstrated that many of the histone deacetylase and methyltransferase genes are down-regulated by one week of voluntary wheel running in conjunction with increased cerebellum and hippocampus H3ac in juvenile mice.[410] Intriguingly, the exercise-induced modulation of epigenetic machinery may be blunted by ageing. At the protein level, Dnmt1 and Dnmt3b were decreased after 20 minutes of treadmill exercise in young rats only.[405] Unfortunately, the elevated stress experienced by rodents forced to swim or run may potentially confound results on epigenetic modifications found in the brain in response to exercise. Nonetheless, these studies demonstrate the existence of epigenetic changes after acute and chronic exercise and show they are associated with improved cognitive function and elevated markers of neurotropic factors and neuronal activity (BDNF and c-Fos). These exercise-induced changes seem to be mediated by DNA methylation and histone H3 and H4 modifications through DNMTs (DNMT1 and 3A/B), HATs and HDACs (eg. HDAC5-8). Further animal model studies, however, are required to elucidate whether epigenetic modifications and underpinning modifying enzymes, are required for the exercised-induced benefits in the brain.

Skeletal muscle

An acute bout of interval-training-like exercise (five sets of 17 minute swimming efforts with a load equivalent to 3% of body weight) caused a paralleled elevation in CAMK-2 phosphorylation, H3ac at the myocyte enhancer factor 2 (*Mef2*) on the glucose transporter-4 (*Glut4*) gene, *Mef2a*, *Glut4* mRNA and protein abundance in rat triceps muscle.[402] The authors suggested the molecular alterations observed might be a result of the exercise induced CAMK-2 activation and subsequently, higher *Glut4* expression, through accessible binding of *Mef2a*, promoting transcription.[402] Notably, others have demonstrated elevated phosphorylated CAMK-2 and epigenetic modifications enhancing transcription after voluntary aerobic exercise in humans,[345,408,411] supporting a role for CAMK-2-mediated chromatin remodelling. Additionally, epigenetic modifications may regulate muscle gene expression through motor unit recruitment, as neuronal activity modulates Mef2, HDACs and histone modifications in mice.[412,413] An alternative potential mechanism may be that the transcriptional changes and epigenetic modifications induced by exercise involves the modulated cytosol calcium content, which has been shown to cause paralleled epigenetic and transcriptional changes in C_2C_{12} muscle cells treated with caffeine.[414]

Genetic predisposition to athletic performance has been the topic of intense investigation, as many single nucleotide polymorphisms (SNPs) and other molecular biomarkers are associated with muscle-growth, endurance performance and trainability.[415-419] DNA methylation was related to muscle growth and endurance performance, with a greater number of genetic polymorphisms associated with enzymes involved in DNA methylation (5,10-methylenetetrahydrofolate reductase, methionine synthase and methionine synthase reductase) found in elite endurance athletes compared to apparently healthy controls.[420] Thus, there may be a heritable predisposition to a less-methylated genetic environment.[420] *In vitro* analysis using murine C_2C_{12} cells with chemically induced DNA-hypomethylation,

confirmed hypomethylation enhances intermediate and late myocyte differentiation, with myocytes and myotubes exhibiting greater myogenic gene expression and cross-sectional area, respectively.[420] Although human myoblasts were not used for *in vitro* analyses, these results add compelling evidence suggesting hypomethylation in differentiating myoblasts may promote muscle growth and maintenance. For further discussion on the role epigenetic modifications in the adaptation to exercise and performance, the readers are referred to other publications.[411,421,422]

Histone HDACs and HATs regulate the extent of histone acetylation and inevitably gene expression.[342] Moderate and high intense aerobic exercise promotes chromatin remodelling and enzymes responsible for acetylation. Sixty minutes of cycling at approximately 75% of VO_{2peak} increased H3K36ac, without H3K9 and H3K14ac in skeletal muscle of young men.[345] These histone modifications were observed in conjunction with decreased nuclear HDAC4 and 5, along with increased AMP-activated protein kinase (AMPK) and CAMK phosphorylation immediately after exercise.[345] Increased phosphorylation of HDAC4, 5 and 7 was observed immediately after high-intense exercise (80% of VO_{2peak}), and was accompanied by phosphorylated AMPK, CAMK, activating transcription factor and acetyl-CoA carboxylase,[423] supporting previous results noting AMPK and CAMK involvement in myocyte chromatin remodelling.[345] Moreover, ameliorated HDAC4 protein abundance were reported in young and older humans six hours following acute resistance exercise in conjunction with altered miRNA dynamics,[424] highlighting the interplay between epigenetic modifications and miRNAs in regulating gene expression. Additionally, in men, the miRNA processing enzymes (Drosha, Dicer and Exportin) and HDAC4 protein abundance was increased with modulated myomiRs, including decreased miR-31, in skeletal muscle of young men after acute aerobic exercise.[425] Interestingly, miR-31 expression was further reduced after 10 days of exercise training.[425] Through luciferase assays, miR-31 was

demonstrated to bind to and reduced *HDAC4* and *NRF1* mRNA expression – two genes responsible for transcriptional regulation.[425] Indeed, other small non-coding RNAs are involved in DNA methylation by targeting of CpG sites and directing DNA methylation.[426,427]

The physical exercise-induced decrease in specific cancer risk and increased longevity is intensity and dose-dependent.[56,269,428-430] Similarly, physiological adaption to physical exercise is proportional to exercise-intensity and volume.[56,431,432] Recently, an exercise-intensity- and dose-dependent relationship was demonstrated at specific promoter regions of genes involved in skeletal muscle metabolism.[344] Acute aerobic-exercise decreased global DNA methylation,[344] an indicator that temporarily modulated transcriptional changes caused by exercise may be required for exercised-induced adaptations. Furthermore, the quantification of DNA methylation at CpG sites related to genes involved in metabolism – peroxisome proliferator-activated receptor-γ, coactivator 1-α (*PPARGC1A*), peroxisome proliferator-activated receptor-δ (*PPARD*) and mitochondrial transcription factor-α (*TFAM*), revealed decreased DNA methylation with paralleled increased gene expression after acute-exercise.[344] The reduction in DNA methylation described was dependent on exercise intensity, with more intense exercise causing greater post-exercise DNA demethylation.[344]

While regular moderate amounts of physical activity confers health benefits, potentially through epigenetic modifications, physical inactivity is detrimental and also may regulate the epigenome.[433,434] As little as nine days of physical inactivity (bed rest) is enough to increase insulin resistance and methylation of *PPARGC1A* CpG promoter region.[434] Remarkably, after short-term physical inactivity, physical aerobic re-training did not fully alleviate the bed-rest-induced DNA methylation.[434] Therefore, both acute exercise [344] and nine days of physical inactivity [434] regulates *PPARGC1A* DNA methylation and gene expression in muscle. These

results also advocate the need for maintaining physical activity and highlight the potentially deleterious short-term epigenetic modifications caused by physical inactivity.

The consequences of physical inactivity to health and disease risk are well known (eg. elevated risk of heart disease, cancer and type 2 diabetes). Interestingly, there are differences in the resting skeletal muscle DNA methylation profile of individuals with and without a family history of type 2 diabetes mellitus.[435] Furthermore, six months of aerobic exercise altered DNA methylation in genes involved in unique pathways amongst men with and without a family history of type 2 diabetes mellitus.[435] Of the 21 candidate genes for type 2 diabetes mellitus two (*THADA* and *RBMS1*) were significantly down-regulated by aerobic exercise.[435] Six months of aerobic exercise training also changed whole-genome DNA methylation in adipocytes.[436] Specifically, the aerobic exercise training induced changes in DNA methylation in 24 and 45 CpG sites, corresponding to 18 candidate genes for obesity and 21 for type 2 diabetes mellitus, respectively.[436] Importantly, the authors performed luciferase assays and small interfering RNA (siRNA) *in vitro* experiments to demonstrate the exercise-induced increased DNA methylation in the CpG sites relating to the promoter regions of *RALBP1*, *NCOR2* and *HDAC4* down-regulated gene expression and consequently increased lipogenesis.[436] This novel study has provided compelling evidence that suggests adaptations conferred by exercise involves (to a certain extent) epigenetic modifications.

Collectively, the acute and chronic (six months of aerobic exercise) aerobic exercise training induced changes to skeletal muscle DNA methylation have been studied. While the effect of acute aerobic exercise on specific CpG sites in some genes are known, there is scope for the profiling of acute exercise-induced changes to whole-genome DNA methylation. Moreover, acute aerobic exercise influences H3ac,[345,402] but whether these histone modifications and other epigenetic changes directly influence, or are a response, to the

exercise-induced changes to phenotype requires explanation. Finally, the analysis of epigenetic changes in skeletal muscle caused by resistance exercise training is also warranted.

Peripheral blood

Physical activity and aerobic training alters DNA methylation status of leukocytes.[194,343,437-441] Immune system dysfunction can result in chronic low-grade inflammation, which is a hallmark characteristic shared by numerous diseases, including cardiovascular disease, cancer, autoimmune disorders and ageing.[312,442-444] Regular engagement in aerobic exercise training is associated with the lowering of basal levels of inflammation [280,445,446] and the mechanisms of this therapeutic effect may be mediated by DNA and chromatin remodelers.

The majority of the human genome is methylated,[355,356] and loss of leukocyte DNA methylation is observed with ageing.[340,447] Furthermore, loss of DNA methylation and other aberrant DNA methylation signatures are involved in numerous disease pathophysiology and development.[448,449] Methylation of promoter pro-inflammatory and apoptotic gene, apoptosis-associated spec-like protein containing a caspase recruitment domain - *PYCARD* (*ASC*), declines with age.[439] Six-months of moderate-intense aerobic training, however, attenuated the age-related loss of DNA methylation at CpG islands (exon 1) within the *ASC* gene in older adult circulating leukocytes.[439]

Sub-telomeric DNA methylation is important for maintaining telomere dynamics and chromosomal stability. Telomeres are the genetically conserved repeated DNA sequences capping the ends of our chromosomes [67] and are implicated in ageing, chronic disease, mortality risk.[20,450] Lifestyle factors, such as regular participation in aerobic endurance exercise, is associated with the attenuated telomere length attrition associated with ageing.[451]

Physical capacity and improvement in physical capacity is positively correlated with sub-telomeric DNA methylation and with longer telomeres in previously deconditioned older-adult patients with cerebrovascular disease.[194] Tai Chi practitioners exhibit ameliorated epigenetic age-related DNA methylation changes at six specific CpG sites (two located at subtelomeric regions) compared to apparently healthy individuals.[343] For additional comprehensive discussions on exercise-induced epigenetics modifications in relation to ageing and disease, the reader is directed elsewhere.[452-454] While there seems to be a relationship between DNA methylation changes with ageing, whether these are blunted or prevented by exercise requires further clarification.

Notably, those who exercised moderately, defined by 26-30 minutes per day (measured with accelerometers), demonstrated greater global leukocyte DNA methylation (LINE-1) compared to those who engaged in less and more physical activity.[437] Self-reported physical activity levels during childhood, adolescence and in the 12 months before sample collection was positively associated with leukocyte LINE-1 DNA methylation in women.[455] Conversely, physical activity (assessed by questionnaires) was not correlated to LINE-1 and *IL-6* promoter-specific leukocyte DNA methylation. [438] Also, average global DNA methylation was inversely related to physical activity levels in older adults (>70yrs).[456] Therefore, additional studies will help validate whether exercise may prevent the risk of cancer by mechanisms involving increased LINE-1 DNA methylation, as loss of LINE-1 DNA methylation is associated with cancer and carcinogenesis.[457,458] Physical activity seems to be constructive at ameliorating risk of certain cancers, and it is positively associated with mitigating dysfunction DNA methylation related to cancer.[459-462] A stand-alone, randomized clinical trial demonstrated 43 genes were differentially expressed as a result of DNA methylation alterations induced by a six month, moderate-intense aerobic training intervention.[440] A link was observed between reduced *L3MBTL1* DNA methylation,

increased *L3MBTL1* gene expression in blood and tumour samples, and patient survival.[440] They suggested peripheral blood DNA methylation may be a clinically relevant biomarker and reflective of epigenetic changes in other tissues.[440]

DNA methylation changes may be a useful tool for monitoring exercise training interventions. Participant VO_{2max} and physical activity levels were correlated to buccal cell DNA methylation at a number of CpG sites located in candidate genes for breast cancer.[463] Moreover, average buccal cell DNA methylation, quantified at over 27,000 CpG sites, significantly increased after a 12 month exercise promotion intervention.[463] Furthermore, an epigenetic score calculated from leukocyte DNA methylation of 97 CpG sites after a 10-week multi-disciplinary health intervention (encompassing physical training, as well as diet and psychological support), related to decreases in body mass index.[464]

Together, these studies identified the effects of physical activity levels and long-term (>12 months) exercise training has on leukocyte DNA methylation. Leukocyte DNA methylation changes caused by ageing may be somewhat attenuated by aerobic exercise training, but require further study. Notably, the acute effects of exercise on leukocyte DNA methylation are yet to be reported. Investigations into histone modifications in leukocytes after exercise are also yet to be studied. Notably, epigenetic changes caused by exercise in differentiated leukocytes are warranted to elucidate the specific biological impacts of these on cell-specific functions. Finally, identifying the sensitivity and the DNA methylation signatures altered by exercise training will further highlight its usefulness in monitoring exercise training.

In summary, short and long-term exercise training results in dynamic changes to DNA methylation and histone modifications, in a variety of tissues. To our knowledge, the effects of resistance exercise training on epigenetic modifications have not yet been reported.

Delineating the cause or effect, relationship between exercise-induced adaptation to phenotype and epigenetic changes will be a challenge for future studies.

Are exercise-induced epigenetic modifications trans-generationally inherited?

Certain epigenetic modifications are passed on to subsequent generations and affect gene expression.[59,465,466] Not only does the maternal and paternal environment impact on epigenetic modifications and gene expression, but it also influences that of the offspring. Environmental stimuli, such as endocrine disruptors [58,467] and various diets [466,468,469] causes distinct inheritance of epigenetic modifications, which have also been shown to be trans-generationally heritable.[59,467,469] For example, a maternal protein restricted diet throughout pregnancy produced lower hepatic *Ppara* promoter DNA methylation across the next two generations of rats.[469] Similarly, paternal low protein diet from weaning until sexual maturity caused increased *Ppara* enhancer-region DNA methylation and markedly altered gene expression in mouse offspring compared to normal diet controls.[59] Late-gestational calorie restriction in mice caused decreased placental DNA methylation and transcript expression in genes enriched for metabolic and cardiovascular system developmental pathways when compared to mice fed a normal chow diet.[470] In humans, paternal obesity was inversely correlated with DNA methylation at three CpG sites upstream of exon 3 of the imprinted *IGF2* gene from umbilical cord blood of new-borns.[471] Notably, physical exercise modulates gene expression and DNA methylation of many metabolic genes affected by diet, including some of the peroxisome-proliferator activated receptor-associated genes.[344,435] Physical exercise may be another environmental stimulus shaping epigenetic modifications and gene expression of not only the individual performing the physical exercise, but also their progenies'.

The influence of exercise on miRNA expression

Contrasting epigenetic mediators, miRNAs also regulate gene expression and protein abundance via post-transcriptional modifications.[349] Of the hundreds (>1000) of miRNA currently known, a relatively small subset are responsive to acute [472,473] and chronic [474,475] physical exercise (refer to Electronic Supplementary Material Table S1 for a comprehensive outline of miRNAs responsive to physical exercise).

Central nervous system

The brain and spinal cord are highly plastic tissues. Damage to either organ can result in potential catastrophic and physically debilitating conditions. Aerobic exercise positively regulates neurotrophic factors and has been implicated in the rehabilitation of the central nervous system.[476,477] Due to the highly invasive nature of collecting neuronal samples or brain tissue, human studies are not feasible. Therefore, rodents have been used. Interestingly, miRNAs have recently been implicated in spinal cord rehabilitation. Spinal-cord-injury-associated inflammation and apoptosis was attenuated via reduced spinal cord miR-15b and elevated miR-21 after five, but not 20 days of aerobic exercise in rats.[478] Therefore, initial exercise may be pivotal to preventing spinal cord injury-associated apoptosis and this may be in turn regulated by phosphatase and tensin homolog (Pten)/ mammalian target of rapamycin (mTor) signalling.[478] Although these results are intriguing, tissue samples were taken one hour after the last training session, suggesting the authors were measuring the adaptation to acute response to exercise, which may not be as essential to rehabilitation as the overall training adaptation of miRNAs measured at rest. Nonetheless, attenuated spinal cord miRNA dynamics by exercise is possible and warrants further investigation. The aerobic exercise training induced changes to miRNA profile in the brain seems to be intensity-dependent.[479]

These few studies provide basis for further exploration into potential miRNAs involved in brain and neuronal development and recovery via aerobic exercise.

Cardiovascular system

Engaging in regular aerobic exercise causes a concomitant increase in non-pathological left ventricular hypertrophy that can be concentric or eccentric, depending on the mode of exercise and miRNAs are involved (for detailed review see papers by Fernandes *et al*. [480] and Gielen *et al*. [481]). Contrasting pathological cardiac hypertrophy, non-pathological cardiac hypertrophy induced by physical exercise involves increased sarcolemma adjacent and in series in the heart muscle, without clinical molecular biomarkers (atrial natriuretic factor or perturbed skeletal muscle α-myosin heavy chain to β-myosin heavy chain ratio).[480] This contributes to greater heart contractile capacity and subsequently cardiac output, cardiopulmonary efficiency and exercise performance. A ten-week aerobic training intervention caused a dose-dependent reduction in miR-1, -133a and -133b and increased miR-29a and -29c paralleled with cardiac hypertrophy in rats,[482] results which were reproducible in different rats.[483] Whether these miRNAs directly affect expression of genes related to the renin-angiotensin system, such as – the angiotensin converting enzyme (*Ace*) and *Ace2*, thereby causing cardiac hypertrophy, is unknown.[483] Moreover, others have shown a role for miRNAs – miR-21, -124 -144 and -145 in phosphoinositide-3-kinase catalytic-α polypeptide (Pik3a)/Akt/mTor signalling pathway associated non-pathological cardiac hypertrophy after chronic swimming exercise in female rats.[484]

Dysfunctional angiogenesis is observed in patients with arteriosclerotic disease.[485] Aerobic endurance training, however, promotes angiogenesis, which may be, in part, regulated by miRNAs. Treadmill running re-establishes normal expression of miR-16, -21

and -126 in the spontaneously hypertensive rat (SHR), increases biomarkers of vascularisation and decreases blood pressure.[486] miR-126 is paramount for maintaining endothelial integrity, as deletion causes severe endothelial cell dysfunction.[487,488] _ENREF_21 Furthermore, angiogenesis is enhanced by aerobic exercise-induced elevation in miR-126 expression, leading to decreased Spred-1 protein and *Pi3kr2* mRNA abundance by regulating mitogen-activated protein kinase 1 (MAPK) and PI3KR2 signalling pathways.[489] These novel findings suggest miR-16, -21 and -126 as targets for blood pressure control and vascular functioning in cardiovascular disease. Notably, miR-21 expression increased after chronic exercise training and was related to non-pathological cardiac hypertrophy,[484] as well as blunting of endothelial cell dysfunction and hypertension.[486] Therefore, regulation of miR-21 may be one mechanism by which exercise provides favourable benefits to cardiovascular health and performance. Whether miRNAs are manipulated by exercise to augment lower resting blood-pressure in hypertensive humans remains to be determined.

Skeletal muscle

Resistance exercise

Acute resistance exercise causes anabolic gene expression, increasing myocyte transcription factors and protein synthesis.[490-492] By identifying the pathways and molecular mechanisms regulating these muscle growth factors, we may not only be able to ameliorate muscular pathologies (eg. muscular dystrophies and sarcopenia), but enhance rehabilitation efficiency and sporting performance. Studies have implicated miRNAs in muscle anabolic mechanisms,[424,493-495] such as miR-1, -26a, -29a, -133a, -378 and -451. Additional studies,

however, are required to validate and further establish the functional biological relevance of these miRNAs.

It is well known that miRNA expression is cell type-specific, and this is consistent with a novel study that indicated differential basal miRNAs and pri-miRNAs expression between mouse soleus and plantaris muscles.[495] In mice, muscle under mechanically-induced functional overload increased expression of pri-miR-1-2, 133a-2 and -206 after seven days, with concomitant increases in the activity of miRNA-processing enzymes Drosha and Exportin-5, but not Dicer.[495] Along with the observed changes to miRNAs and processing enzymes, there was a 45% increase in plantaris muscle wet-weight.[495] These early results emphasise miRNAs are muscle specific, and their involvement in skeletal muscle hypertrophy. Furthermore, these data provide insights into miRNA biogenesis enzyme activity, during hypertrophic stimuli.

Sarcopenia is an age-related disease, involving the muscle atrophy and the laying down of connective tissue. Indeed, the ability to maintain lean muscle mass is hindered with ageing. Similarly, miRNA dynamics are also disturbed with ageing.[496,497] In humans, young and older adults' pri-, pre-miRNA expression and miRNA biogenesis enzyme mRNA (Exportin-5 and Drosha) expression differs in response to acute resistance exercise with complementary leucine-enriched essential amino acid ingestion.[424] Interestingly, myocyte miR-1 expression was reduced in young adults and was unchanged in older adults resulting from leg extension resistance exercise (8 set of 10 repetitions with 70% of one repetition maximum (1RM)) with essential amino acid ingestion.[424] miR-1 is a muscle-specific miRNA and a well-defined facilitator of muscle cell growth and regeneration.[498-500] _ENREF_30Perplexingly, there are no clear patterns of miR-1 expression in response to resistance and aerobic exercise training. Twelve weeks of resistance training attenuated basal miR-1 expression in older adult human volunteers.[494] In young men, however, basal miR-1 expression was unchanged following a

similar 12-week resistance training intervention.[493] Acute aerobic endurance exercise up-regulates miR-1 expression,[425,475,501] and is subsequently down-regulated at basal levels in healthy males after 12 weeks of aerobic exercise training.[475] Others, have found increased basal miR-1 and miR-29b expression after 10 days of aerobic exercise training.[425] Conversely, miR-1 expression is attenuated after seven days of bed-rest and unchanged following acute one-legged knee extension exercise in men.[433] The function of miR-1 in the adaptation to exercise, is therefore, unclear, but may be delineated with additional functional, animal and *in vitro* studies.

Through the analysis of SNPs and gene expression profiles, researchers have differentiated between high and low responders to aerobic exercise, suggesting that genes are correlated to physical performance.[415-417,502,503] In an attempt to distinguish genetic predisposition and anabolic response to resistance exercise using miRNAs, young adults were divided into low and high responders according to gains in lean muscle mass and muscle cross-sectional area.[493] Of the 21 miRNAs investigated, miR-378 and -29a were lower and miR-451 was higher in low responders compared to the high responders.[493] Additionally, miR-378 was significantly correlated with gains in lean body mass.[493] Sedentary behaviour also induced expression changes in subsets of miRNAs (lower miR-1 and -133a,[433] miR-107, -221 and -499 [504]). Importantly, miR-499 was validated by luciferase transfection assays to target the transcription factor, *Sox6*.[504] These results provide evidence supporting the involvement of miRNAs in regulating skeletal muscle phenotype through physical inactivity and exercise training. Identifying other miRNAs differentially expressed in high and low responders to exercise and discovering how these miRNAs function and influence phenotype are likely to be the next step.

Aerobic exercise

Data from miRNA arrays has suggested moderate-intense aerobic exercise induces the differential expression of numerous miRNAs, involving pathways such as regulation of transcription, metabolism, cell and muscle development and other cellular processes.[417] The regulation of metabolic genes involved in disease and endurance performance are also mediated by miRNAs. For example, expression of miR-23, predicted to target *Ppargc1a* – the transcriptional co-activator and positive regulator of genes involved in mitochondria activity, glucose and lipid metabolism – is decreased in conjunction with higher *Ppargc1a* mRNA and protein abundance, in response to prolonged aerobic exercise in mice.[501] Similarly, *Ppargc1a* miRNA regulating candidate, miR-696, was lower in mouse skeletal muscle after four weeks of progressive aerobic exercise training and higher after unilateral hind-limb immobilisation.[505] Cultured myocyte experiments subsequently validated miR-696 as a negative regulator of Ppargc1a protein abundance in C_2C_{12} myoblasts.[505] Whether the stress associated with hind-limb suspension mediated the change to miR-696 is unknown, as stress can modulate miRNA dynamics.[506] *Ppargc1a* is a transcriptional co-activator controlling exercise induced angiogenesis,[507] muscle fibre type,[508] and mitochondrial biogenesis[509,510] and for this reason it is a gene doping candidate for enhancing sporting performance._ENREF_38[511] Downstream molecules of *Ppargc1a* signalling cascade (PDK4 and COXII) are targeted by miR-696 which decreases after aerobic exercise training in mice, thereby contributing to increased fatty acid oxidation and mitochondrial biogenesis.[505] Moreover, through luciferase and *in vitro* knockdown experiments, miR-494 was validated to target *Tfam* and forkhead box j3 (*Foxj3*).[505] Acute aerobic endurance exercise increased Tfam and Foxj3 protein abundance, along with increased *Ppargc1a* expression and mitochondrial biogenesis, through lowering miR-494 expression in mice.[512]

The miRNAs biogenesis is regulated by a subset of enzymes with specific roles involved in producing a functional miRNA (Figure 1). Drosha, DGCR8 and Dicer, are unaltered three hours after an acute prolonged bout of aerobic exercise.[501] Drosha and Exportin-5 are, however, increased in response to mechanically-induced functional overload, without changes to dicer content, in mice.[495] Therefore, further studies may elucidate how the miRNA processing enzymes govern miRNA dynamics with exercise training.

The acute, exercise-induced effect of aerobic and resistance exercise is indicative of an immediate damage-response, which in turn produces numerous molecular cascades to inevitably cause a super-compensation and adaption to the exercise stimulus. This may consequently alter resting miRNA/gene expression profiles and blunt the physiological response to the initial physical exercise stimuli. A 12-week aerobic endurance training intervention caused an ameliorated response to acute exercise-induced miR-1 and -133a expression.[475] Moreover, basal miR-1, -133a/b and -206 expression were also reduced as a result of the aerobic training intervention.[475] The reduced basal miRNA expression, however, returned to the pre-exercise profile following two weeks without structured physical exercise training.[475] The attenuated miRNA expression may have been a result of increased VO_{2max} and more favourable body composition observed in participants.[475] Furthermore, there may be a direct relationship between histone deacetylase activity and miRNAs. In men, the miRNA processing enzymes (Drosha, Dicer and Exportin) and HDAC4 protein abundance was increased in conjunction with modulated myomiRs, including decreased miR-31, in skeletal muscle of young men after acute aerobic exercise[425] Interestingly, basal miR-31 decreased after 10 days of exercise training and it was demonstrated to target and subsequently reduced *HDAC4* and *NRF1* expression – two genes responsible for transcriptional regulation – *in vitro*.[425] Skeletal muscle biopsies are invasive and sample collection across multiple time-points is sometimes not feasible. Trends, however, exist

between aerobic exercise performance and expression of specific miRNAs from circulating blood and their use as biomarkers.

Aerobic exercise influences miRNAs in blood

The miRNAs from plasma and serum are not only indicative of immune system function, they have been proposed as biomarkers of physical fitness and performance because they originate from numerous tissues and are mobilised into peripheral circulation. Plasma miR-146a and miR-20a predict VO_{2max} and the trainability of VO_{2max}, respectively.[474] Similarly, serum miR-21 and miR-210 are inversely related to VO_{2max} and are more highly expressed in individuals with categorically low VO_{2max} compared to those with a high VO_{2max}.[513] It was also found that miR-210 expression alone or combined with miR-21 accounted for 12% and 15% of the variation in 100 individuals' VO_{2max} values, respectively.[513] Serum miR-486 was down-regulated after acute and 4 weeks of three times a week cycling and the change in expression was negatively correlated to VO_{2max}.[514] Therefore, miRNAs from blood may be useful biomarkers for exercise capacity and trainability. Plasma miRNAs have been implicated as biomarkers of health and disease risk.[515] Notably, to date, no one has investigated whether physical exercise can attenuate dysfunctional miRNA biomarkers of human disease. Using the miRNAs, miR-126 and miR-133, as surrogate biomarkers for endothelial cell and muscle cell damage, respectively, the effects of different exercise modes were investigated.[516] Interestingly, while miR-126 expression was increased after both a maximal cycle ergometer VO_{2max} test (by 2.1-fold) and a four hour cycle at 70% of VO_{2max} (4-fold), miR-133 expression increased after resistance exercise training (2.1-fold).[516] After a marathon, however, both miR-126 and miR-133 expression increased dramatically (3.4-fold and 8.9-fold, respectively).[516] Although the authors could not account for the potential effects that gender and fitness levels (or physical activity levels) of participants in each of the exercise group had, these results suggest a role for miRNAs in monitoring adverse effects

caused by acute-exercise. Additionally, positive relationships have been identified between specific miRNAs and cardiovascular risk factors – C-reactive protein (miR-21), aspartate aminotransferase (miR-210), Finish Type 2 Diabetes Risk Score (miR-21) and age (let-7d and miR-103).[513] Additionally, replication studies are required to reproduce miRNA responses to exercise, to further establish their relevance as biomarkers.

The miRNA profile of differentiated white blood cells represent genes involved in immune processes, cell adhesion and cytokine production, and are more reflective of vascular health and immunity. It was reported that individuals who completed 30 minutes of intermittent (2 minute) bouts of cycling at approximately 76% of VO_{2max} exhibited altered expression of 38 miRNAs, potentially targeting 4,724 genes in neutrophils.[517] Additionally, 34 miRNAs were altered by the same exercise protocol, including six similarly expressed between neutrophils and leukocytes.[472,517] Although the authors validated four and seven miRNAs in the consecutive studies, respectively, further studies are required to validate their miRNA mRNA targets. Finally, results from the same laboratory revealed 23 modulated miRNAs and gene expression patterns in natural killer cells as a result of acute aerobic exercise.[473] Specifically, gene pathways altered included those involved in type 1 diabetes mellitus, prostate cancer, chemokine signalling, leukocyte trans-endothelial migration and p53 signalling pathway.[473] Interestingly, after acute aerobic running exercise at 80% of VO_{2max}, expression of miRNAs – miR-21-5p, -24-2-5p, -27a-5p and -181a-5p were increased in National-level ski athletes' leukocytes.[518] *In silico* prediction was employed and showed enrichment for genes involved in metabolic pathways, immune response, transcriptional regulation and apoptosis.[518] Finally, acute resistance exercise increased serum miR-149* and decreased miR-146a and miR-221 expression three days after exercise, which along with other miRNAs were correlated to growth hormone, IGF-1 and testosterone concentration.[519]

Interestingly, the resistance exercise-induced change to miR-146a and -221 trended in the opposite direction to that observed after acute aerobic exercise in plasma.[474]

Thus, a number of blood miRNAs have been correlated to VO_{2max} and VO_{2max} trainability. Similarly, expression of specific miRNAs are associated with cardiovascular disease risk factors and adverse effects of acute exercise. Their validity and reliability for biomarkers of VO_{2max} and adverse risk factors will require further testing. Furthermore, whether unique miRNA dynamics are perturbed with overtraining or overreaching is unknown. Modulated miRNAs in blood and other tissues will need to be validated and subsequently functionally tested in order to reveal their biological function and make clear interpretations of how they regulate a phenotype through exercise training.

Conclusions and future directions

Epigenetic modifications and miRNAs dynamics become dysfunctional with disease and ageing. While the current evidence supports physical exercise as a modulator of histone acetylation, DNA methylation and expression of various miRNAs, the effects other epigenetic modifications (eg. histone methylation, sumoylation, deimination and ubiquitylation) are left for investigation. These gene expression regulators seem to be intensity- and volume-specific. Apart from a single study analysing miRNAs in serum and those analysing skeletal muscle, the effects of resistance exercise on miRNAs in human blood cells and other types of tissues are unknown. Similarly, the effect of resistance exercise on epigenetic modifications in human tissues requires further study. Few studies have validated 'predicted' gene targets and demonstrated a biological function of miRNAs influenced by physical exercise in humans. Data on the effect of exercise on miRNA or epigenetic modifications in clinical or athletic human populations is sparse. Further studies may provide us with molecular (epigenetic, miRNA and gene expression) signatures specific to health benefits conferred by exercise, as well as attenuation of disease. More data on the

time-course of epigenetic and miRNA changes caused by exercise are required. Additionally, identification of novel molecular biomarkers in the form of miRNAs and epigenetic modifications may be suitable for monitoring training interventions. The comprehensive study of DNA methylation and other epigenetic modifications in various tissues in response to different modes and volumes (intensity, frequency and duration) of exercise will aid our understanding of the ability of exercise to influence our epigenome. Finally, animal model experiments will help determine the cause or effect relationship between epigenetic modifications and miRNAs with exercise-induced adaptations. The emerging field of exercise epigenomics is expected to prosper and further studies may elucidate the clinical relevance of miRNA and epigenetic modifications, and demonstrate how exercise benefits health and physical performance.

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8.0 Tables

Table I. Definition of key terms.

Chromatin	The elongated and condensed form of DNA housed inside the cell nucleus.
DNA methylation	Epigenetic modification encompassing the addition of a methyl group to DNA CpG and non-CpG sites.
DNA methyltransferase	Actively performs and catalyses DNA methylation. The methyltransferase enzymes include DNMT1, 3A, 3B and 3L.
Epigenome	All epigenetic modifications including DNA methylation and numerous histone modifications described elsewhere.[342] These epigenetic modification all share common characteristics, specifically, they act on DNA or proteins associated with DNA packaging, to manipulate their structure. In doing so, these mechanisms either encourage or repress transcription, depending on the location of the modification.
Histone acetylation	The chemical reaction involving the addition of an

	acetyl molecule to histone proteins.
Histone acetyltransferase	Family of enzymes that catalyse histone acetylation.
Histone deacetylase	Family of enzymes involved in the removal of acetyl groups from histone proteins.
Histone proteins	Constitutes five histone proteins (histone H1, H2A, H2B, H3 and H4) involved in DNA packaging. Four paired histone proteins, H2A, H2B, H3 and H4, form an octamer and contributes to the formation of a nucleosome.
Lysine	Protein sites vulnerable to acetylation and methylation changes along histone proteins.
microRNA	Small non-coding RNA molecule, approximately 18-24 bases in length.
Nucleosome	Repeated structural unit for DNA packaging consisting of eight histone proteins.

Table II. Summary of relevant literature on DNA and histone modifications with physical exercise

Cell type	Participant or animal phenotypes[a,b,c,d,e]	Exercise[f,g,h,I,j]	Main finding	Reference
Human studies				
Leukocytes	647, 35-74, F	N/A	Highest physical activity level across three time-points associated with increased LINE-1 DNA methylation.	White et al. [455]
Leukocytes	509, 70, M+F	N/A	Inverse relationship between physical activity levels and global DNA methylation.	Luttropp et al. [456]
Adipocytes	15 (FH^+) Vs 16	Chronic, aerobic	Changes to DNA methylation of 17,975 CpG sites in response to 6 months	Rönn et al. [436]

	(FH^-) of T2D, 37.3, M, SED, 33.1±4.6	(cycle and aerobics), 60, ~2, 6□	of exercise.	
Myocytes	15 (FH^+) Vs 13 (FH^-) of T2D, 37.5 Vs 37.5, M, SED, 32.0±3.5 Vs 33.0±5.3	Chronic, aerobic (cycle and aerobics), 60, ~2, 6□	Discordant DNA methylation between men with and without a family history of type 2 diabetes mellitus and unique DNA methylation changes with intervention.	Nitert et al. [435]
Leukocytes	24 obese individuals (12 high and 12 low responders), 13-16, M+F	10-week Multidisciplinary weight loss intervention	Differentially DNA methylation at 96 CpG sites between high and low responders after weight loss intervention.	Moleres et al. [464]

Buccal cells	‡37 cases Vs 27 controls, 30.0 Vs 28.4, M+F, SED, 33.6 Vs 32.6	Chronic, exercise promotion intervention [520], ~30, ~5, 12□	Average DNA methylation of over 27,000 CpG sites was significantly increased following intervention.	Bryan et al. [463]
Buccal cells	‡237 cases Vs 263 controls, 64.6 Vs 62.6, F, RA (tai chi) Vs U	N/A	Differential DNA methylation over six CpG (two subtelomeric) sites related to ageing in Tai Chi practitioners versus healthy controls.	Ren et al. [343]
Leukocytes	165, 18-78, M+F	N/A	No relationship between physical activity and LINE-1 for *IL-6* promoter DNA methylation.	Zhang et al. [438]
Tumour cells	‡6 cases Vs 6 controls, U, F, SED	Chronic, aerobic, 15-30, 3-5, 6□	Exercise intervention altered DNA methylation of 43 genes and of these; six were associated with patient survival.	Zeng et al. [440]

Myocytes	14, 25, M+F, SED, 45.2	Acute, aerobic (cycle VO_{2max} test)	Exercise intensity-dependent decrease in global and metabolic gene-specific promoter DNA methylation.	Barrès et al. [344]
	8, 24, M, SED, 40.2	Acute, aerobic cycle (at either 40% or 80% of VO_{2max})		
Leukocytes	85, 45.4, M+F	N/A	DNA methylation at two *COMT* promoter regions associated with genotype and physical activity habits.	Lott et al. [441]
Leukocytes	77 cases Vs 54 controls, 24.8 Vs 32.3, U, athletic (cases) SED (controls)	N/A	Unique polymorphism frequencies in genes coding enzymes responsible for DNA methylation in elite athletes.	Terruzzi et al. [420]

Leukocytes	131, 40<, M+F	N/A	26-30 minutes of moderate-intense physical activity per week associated with greater global DNA methylation (LINE-1) compared to more or less physical activity.	Zhang et al. [437]
Leukocytes	23, 77.7, F	N/A	Physical capacity correlated with sub-telomeric DNA methylation and percentage of longer telomeres.	Maeda et al. [194]
Leukocytes	Case (old exercising) Vs young control Vs old control, 230 Vs 153 Vs 34, M+F, U, 23.8 Vs 23.8 Vs 41.5	Chronic, aerobic (walking interval training), 52.2, ~4, 6□	Marked increase in *ASC* exon 1 CpG island DNA methylation in older adults after six month intervention.	Nakajima et al. [439]

Myocytes	20, 25, U, 43.5	9 days of bed rest followed by aerobic retraining (chronic), cycling (70% of VO_{2max}), 30, 6, 4	Four weeks of aerobic exercise training unable to completely alleviate the increased *PPARGC1A* promoter DNA methylation after 9 days of bed rest.	Alibegovic et al. [434]
Myocytes	9, 23, M, U, 41	Acute, aerobic (cycling at ~75% of VO_{2max}), 60	Increased global H3K36ac and removal of HDAC4 and 5 from the cell nucleus after exercise.	McGee et al. [345]
Breast epithelial cells	45, 43, F	N/A	No statistically significant correlation between physical activity and promoter DNA methylation of tumour suppressor genes – *APC* and *RASSF1A*	Coyle et al.

Brain	Wistar rats, 3 (young) and 20 (old) months, M	Acute and chronic, aerobic (running at 60% of VO_{2max}), 20mins, 7, 2	Differential effects of acute and chronic exercise-induced on changes to H3K9meth and levels of DNMT1 and 3B in young and old rats.	Elsner et al. [405]
Brain	C57BL/BJ mice, 46 (days), M	Chronic, aerobic (wheel-running), U, 7,1	Increased H3ac and modulated gene expression of DNMTs and HDACs in cerebellum and hippocampus after exercise training.	Abel et al. [404]
Brain	Sprague-Dawley rats, ~3 months, M	Chronic, aerobic (wheel-running), U, 7,1	Reduced DNA methylation and increased H3ac in the *Bdnf* promoter region in exercised rats.	Gomez-Pinilla et al. [408]

Brain	Wistar rats, 2-3 months, M	Acute and chronic, aerobic (treadmill running), acute and chronic – 20 (incremental treadmill exercise), 7 (chronic), 2 (chronic)	Decreased global HDAC activity and higher HAT activity at H4 but not H3 after acute but not chronic exercise.	Elsner et al. [409]
Brain	Sprague-Dawley rats, U, M	Chronic, aerobic (wheel running)	Parallel Increased H3-phospho-ac and c-Fos^{+} neurons, coping in response to stress and improved learning strategies in exercised rats two hours after novel environmental change and after forced swimming.	Collins et al. [403]

Myocytes	Wistar rats, U, U	Acute, aerobic (resisted swimming), 5 sets of 17mins with 3 minutes rest between sets	Increased H3ac near *Mef2* bending site of *Glut4* promoter region.	Smith et al. [402]
Brain	Wistar, U, M	Acute, aerobic (swimming)	Increase H3K14ac and c-Fos^{+} neurons in exercised rats, peaking two hours after exercise.	Chandramohan et al. [346]

[a] = Number of subjects or breed of rodent.

[b] = Age of subjects (years).

[c] = Sex of subjects – Male (M) /female (F) or both genders (M+F)

[d] = Physical activity level – sedentary (SED), recreationally active (RA), athletic, unspecified (U).

[e] = maximal aerobic fitness (VO_{2max}) measured in $ml \cdot kg \cdot min^{-1}$.Type of exercise intervention – acute, chronic or acute and chronic exercise.

[f] = Type of exercise intervention – acute, chronic or acute and chronic exercise or not applicable (N/A).

[g] = Mode of exercise – aerobic or resistance exercise.

[h] = Minutes of exercise training per session.

[i] = Day/s or sessions of exercise per week.

[j] = Weeks (or months□) of exercise training (if any).

‡ = Indicates data from individuals from which the main findings originate from.

N/A = Not applicable; LINE-1 = long interspersed nuclear element-1; FH^+ = With a family history of; FH^- = Without a family history of; T2D = Type 2 Diabetes Mellitus; ~ = indicates approximately; Vs = compared to; U = unspecified/unknown data; CpG = cytosine-guanine dinucleotide; LINE-1 = long interspersed nuclear element-1; *IL-6* = interleukin-6; COMT = catechol-O-methyl transferase; *ASC* = apoptosis-associated spec-like protein containing a caspase recruitment domain; DNMT = DNA methyltransferase; H3K36ac = histone-3 lysine-36 acetylation; HDAC – histone deacetylase; HAT –

histone acetyltransferase;APS – Adenomatous Polyposis Coli; RASSF1A – as Association (RalGDS/AF-6) Domain Family Member 1; H3K14ac – histone 3 lysine 14 acetylation; *PPARGC1A* – peroxisome proliferator-activated receptor gamma coactivator-α; *AMPK* = protein kinase, AMP-activated, α-1 catalytic subunit; *Mef2* = Myocyte enhancer factor 2; *Glut4* = solute carrier family 2 (facilitated glucose transporter), member 4. HDAC – histone deacetylase; H3K9meth – Histone 3 lysine 9 methylation; c-Fos^{+} = c-Fos-positive.

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Changes in the Leukocyte Methylome and its Effect on Cardiovascular Related Genes after Exercise

Running head: Exercise Changes the DNA Methylome

Joshua Denham[1], Brendan J O'Brien[2], Francine Z Marques[1], Fadi J Charchar[1]*

[1]Faculty of Science and Technology, Federation University Australia, Ballarat, Victoria, Australia

[2]Faculty of Health, Federation University Australia, Ballarat, Victoria, Australia

*Corresponding author: Fadi Charchar, University Drive, Ballarat, Victoria.

f.charchar@federation.edu.au, Ph: +613 5327 6098, Fax: +613 5327 9242.

J.D was involved in the study design, recruited, tested and trained subjects, performed laboratory experiments, analysed and interpreted data, and wrote the manuscript. B.J.O was involved in the study design, data interpretation and the writing of the manuscript. F.Z.M contributed to the study design, data interpretation and writing of the manuscript. F.J.C contributed to the study concept and design, interpreted data, revised the manuscript, funded and supervised the study.

Abstract

Physical exercise has proven cardiovascular benefits yet there is no clear understanding of the related molecular mechanisms leading to this. Here we determined the beneficial epigenetic effects of exercise after sprint interval training, a form of exercise known to improve cardio-metabolic health.

We quantified genome-wide leukocyte DNA methylation of 12 healthy young (18–24 y) men before and after four weeks (thrice weekly) of sprint interval training using the 450K BeadChip (Illumina) and validated gene expression changes in an extra seven subjects. Exercise increased subjects' cardio-respiratory fitness, maximal running performance and

decreased low-density lipoprotein (LDL)-cholesterol concentration in conjunction with genome-wide DNA methylation changes. Notably, many CpG island and gene promoter regions were demethylated after exercise, indicating increased genome-wide transcriptional changes. Amongst genes with DNA methylation changes, epidermal growth factor (*EGF*), a ligand of the epidermal growth factor receptor known to be involved in cardiovascular disease, was demethylated and showed decreased mRNA expression. Additionally, we found that microRNAs, miR-21 and miR-210 gene DNA methylation were altered by exercise causing a cascade effect on the expression of the mature microRNA involved in cardiovascular function.

Our findings demonstrate that exercise alters DNA methylation in circulating blood cells in microRNA and protein coding genes associated with cardiovascular physiology.

Keywords: Epigenetics, sprint interval training, DNA methylation, cholesterol, miRNA

Introduction

Exercise training can prevent and attenuate symptoms of cardio-metabolic diseases, such as insulin resistance, elevated blood pressure (BP), excess adiposity and vascular impairment [521]. The exact molecular mechanisms underpinning the health benefits gained from regular exercise training, however, are not known. DNA methylation affected by environmental factors modulates gene expression and may therefore mediate the health benefits gained from exercise training [57].

Recently, individuals involved in a six month moderate intensity exercise training program displayed genome-wide skeletal myocyte and adipocyte DNA methylation changes in genes associated with type 2 diabetes [522,523]. Additionally, older adult leukocyte DNA methylation increased in the pro-inflammatory *PYCARD* gene after six months of aerobic exercise training, to levels observed in their middle-aged peers [524]. Although six months of moderate intensity aerobic exercise modulates the DNA methylome, it is unknown whether shorter training regimes impact DNA methylation. Moreover, while leukocytes are known to contribute to vascular disease [525], it is not known whether exercise is an environmental factor that regulates the leukocyte methylome and, in turn, contributes to improved vascular health.

Given the effects of acute exercise on DNA methylation are intensity-dependent [526], we aimed to determine whether leukocyte DNA methylation changes occur after short-term (four weeks) yet intense exercise training. We used sprint interval training as a form of exercise training because it rapidly improves vascular functioning [527,528], insulin sensitivity [529], cardio-respiratory function and physical performance (reviewed in [530,531]). We aimed to: 1) identify the effects of four weeks of thrice weekly exercise on genome-wide leukocyte DNA methylation in healthy young men; 2) establish whether specific DNA methylation changes occurred reciprocally with altered gene expression and; 3) identify the acute effect of maximal exercise on genes and microRNAs (miRNAs) related to cardiovascular disease. We

hypothesised that four weeks of exercise training would cause significant changes in DNA methylation in genes and miRNAs related to pathways involved in cardiovascular health.

Materials and Methods

Participants

Twenty-six healthy young men not already engaged in intense exercise training were recruited for this study. Participants were initially screened for any chronic diseases by health and physical activity readiness questionnaires. During the initial assessment, participants' height, weight and body-mass-index (BMI) were recorded. Resting BP was measured with the subjects seated using an electronic BP monitor (Microlife BP 3AQ1). Subjects' BP was measured after a 10 minute rest and was the average of two measurements broken up by a one minute rest period. Body fat percentage was estimated by summing seven skinfolds as described previously [532].

All subjects gave written informed consent and this study was approved by Federation University Australia's Human Research Ethics Committee.

Cardiopulmonary exercise testing and training

In order to assess cardiorespiratory fitness, participants completed one maximal oxygen consumption ($\dot{V}O_{2max}$) test, on the same day of the initial assessment and 48-96 hours following their final exercise session. Participants began training three days but no longer than one week after their $\dot{V}O_{2max}$ test and completed twelve training sessions (three per week) over four weeks. Participants completed two maximal treadmill exercise ($\dot{V}O_{2max}$) tests before and after exercise training. Before the $\dot{V}O_{2max}$ test participants were fitted with a two-way breathing valve (Hans Rudolph, USA) and expired air was collected into an online metabolic

system (Moxus, USA) for gas (O_2 and CO_2) analysis. The metabolic system was calibrated before each test using ambient air and gas of known composition. After a standardised five minute warm-up, the treadmill speed was increased one kilometre per hour every minute until the participant reached volitional exhaustion. Subject' $\dot{V}O_{2max}$ were determined as the highest oxygen consumption over one minute and was expressed as a relative value in $ml \cdot kg \cdot min^{-1}$ by dividing the $\dot{V}O_{2max}$ by body weight.

So as to restrict any potential DNA methylation changes to the exercise program, participants were requested not to deviate from their normal physical activity and recreational exercise training habits, as physical activity and exercise training habits influence leukocyte DNA methylation [57]. Participants were also requested not to make changes to their normal diet, as dietary changes have been associated with DNA methylation alterations [533,534]. Participants had 48-72 hours recovery before the subsequent training session, to allow adequate recovery.

Before each exercise session, participants were instructed to perform a warm-up involving a brief low-intensity run (5 min), dynamic stretches and some short (20 m) high-intensity runs. Table 1 outlines the training schedule performed. Briefly, participants were required to complete three sprints at maximal intensity. To prevent overtraining, a two-up one-back training model was used. For example, during week one of training, participants completed three sprints on the first session, four sprints on the second session and five on the third and final session of week one. Participants then completed four sprints on the first session of week two (Table 1). The training was progressively overloaded until participants performed eight sprints on the final exercise session. Exercise sessions were monitored and supervised by an Accredited Exercise Physiologists (with Exercise and Sports Science Australia) to ensure the safety of participants. Training was performed on a university athletics oval. Participants were requested to run at maximal intensity for each 30 sec effort. To ensure

participants were running at maximal intensity, participants received constant verbal motivation. Participants began each effort at one end of the oval and individually ran around the oval until a whistle was blown to indicate the 30 sec had been completed. Participants were then given a 4 min rest, which involved a slow walk back to where they had started the test, followed by passive rest.

Blood processing

Participants donated a resting blood sample before and after their initial and final (after four weeks of exercise training) treadmill tests. Participants were asked to refrain from consuming alcohol or caffeinated beverages 24 hours before blood draw and were seated for approximately 30 minutes prior to their donations before and after the exercise intervention. Circulating blood was drawn from the antecubital vein into a serum separating tube and an EDTA tube with participant seated. All resting blood samples were collected from participants in the morning following an overnight fast. The final blood collection was obtained 48-96 hours after the final exercise session. Blood was temporarily stored on ice before further processing. The serum separating tube was left to clot at room temperature before centrifugation (3500 RPM) and was subsequently used to quantify blood lipid concentration using the CHOL2, TRIGL and HDLC3 reagents that were run on the Roche c701 instrument at Melbourne Pathology (Melbourne, Australia). All DNA and RNA were extracted on the same day, within three hours of blood draw to prevent possible *de nova* influences on DNA methylation and gene expression. DNA and RNA were extracted from whole blood stored in EDTA tubes using the PureLink Genomic DNA Mini Kit (Life Technologies) and miRNeasy Mini Kit (Qiagen), respectively, following the manufacturers' recommendations. The whole blood leukocytes were washed twice with the erythrocyte lysis wash buffer (included in the miRNeasy Mini Kit) to isolate leukocytes from plasma, serum,

platelets and lysed erythrocytes. Therefore, only whole blood leukocyte miRNA and gene expression were analysed in our study.

Genome-wide DNA methylation and EpiTYPER assay

While 19 participants completed the exercise intervention, whole-genome DNA methylation analysis was performed in 12 subjects at rest before and after the four week exercise intervention. The 450k BeadChip uses two types of Illumina chemistry to provide a comprehensive DNA methylation status of over 480,000 CpG sites spanning 99.9% of refseq genes. DNA methylation is represented as a β-value which corresponds to the percentage of methylation at a given CpG site. All samples quantified on the BeadChip passed the GenomeStudio Methylation Module's quality control procedure. To identify technical variation and increase the chances of detecting DNA methylation changes caused by exercise, raw β-values underwent Subset-quantile Within Array Normalization (SWAN) [535]. Data was subsequently imported into and analysed using the bioinformatics software, Partek (Genomic Suite, version 6.6, Singapore). All β-values were log-transformed into M-values using the logit function ($\log^2(\beta/(1-\beta))$). M-values are a more valid alternative for detecting DNA methylation changes, as they eliminate the heteroscedasticity of higher- and lower-end β-values [536]. Whereas M-values are more appropriate for analysing DNA methylation, β-values are more suitable for displaying biological relevance, as they correspond to the DNA methylation percentage of an individual CpG site. For this reason, M-values were converted back to β-values for all graphs and tables. Hierarchical clustering and pathway analysis was analysed using the software, Partek (Genomic Suite, version 6.6, Singapore). Due to the uneven CpG distributions between the two chemistries used on the Infinium HumanMethylation450 BeadChip (Illumina) – Infinium I (135,501) and Infinium II (350,076), we analysed these separately. Whole-genome DNA methylation was quantified using an Infinium HumanMethylation450 BeadChip (Illumina) according to the

manufacturer's guidelines and with the assistance of the Australian Genome Research Facility (Melbourne, Australia).

The EpiTYPER (Sequenom) was used to validate the DNA methylation change of the epidermal growth factor (*EGF*, cg12093976) and uracil-DNA glycosylase (*UNG*, cg20982606) genes, and these assays were performed by GeneWorks (Melbourne, Australia). Experiments were conducted according to the manufacturer's procedures. Briefly, 1µg of DNA was bisulfite converted and PCR amplified using the following primer sets: cg12093976: sense, aggaagagagAGTTATAATTTTTGGATTGGGGTTG and antisense, cagtaatacgactcactatagggagaaggctTAATTTAATTTTATCTCCATCCTTCCAA; cg20982606: sense, aggaagagagGATTATTTTGGAGTTGAGGAGGTAG and antisense, cagtaatacgactcactatagggagaaggctCCTTAAAAACCTATCCAAAAAACAA. Base-specific (C and T) cleavage and *in vitro* transcription was performed, followed by mass spectrometry (MALDI-TOF). Data was analysed using the EpiTYPER 1.2 software (Sequenom).

Gene and miRNA expression

The gene and miRNA expression of blood collected before and after initial and final treadmill exercise testing was assessed by qPCR using TaqMan assays (Life Technologies). RNA samples were reverse transcribed using the High Capacity Reverse Transcription Kit and the TaqMan MicroRNA Reverse Transcription Kit for gene and miRNAs, respectively, following the manufacturer's procedures (Life Technologies, Australia). Experiments on 384-well plates comprised of samples, endogenous positive and negative controls, all in duplicate were run on the ViiA 7 Real-Time PCR System (Life Technologies, Australia). Standard TaqMan assay procedures were followed and TaqMan assays used in the experiments are outlined in Table 2. The cycle threshold (Ct) of genes and miRNAs were compared to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and the small-nucleolar RNA, *RNU44*, respectively. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [303] and the

results were graphically represented as fold change. The cycling conditions were as follows: a hold at 50° for 2 min and at 95° for 20 sec, followed by 40 cycles at 95° for 1 sec and 60° for 20 sec. As a quality control, duplicate samples greater than one Ct apart we re-run before being included in the analysis. All qPCR experiments were performed by the same researcher.

Statistical analyses

Participant phenotypes were assessed for normality using Kolmogorov-Smirnov and Shapiro-Wilks tests. Parametric data are expressed as mean ± standard deviation (SD) and non-parametric data are expressed by median (interquartile range). Non-parametric data was log-transformed before further analyses. While paired t-test was used to identify statistically significant changes to phenotypes, repeated measures ANOVA were used to show gene expression changes after four weeks of exercise training using the statistical software, IBM SPSS Statistics (version 21.0). Genome-wide DNA methylation changes in relation to CpG islands and gene regions were assessed using Chi^2 and Wilcoxon matched-pair signed ranked tests. DNA methylation changes caused by exercise training were determined using two-way ANOVA after β-values were log-transformed. In order to control for the discovery of false-positives, a false discovery rate (FDR) correction was applied to the whole-genome DNA methylation *P*-values by converting *P*-values to *q*-values. Whole genome data was analysed using the software, Partek Genomic Suite (version 6.6). Gene and miRNA expression were analysed using a two-way repeated measures ANOVA. The miRNA-mRNA targets were predicted using the miRWalk website [537], which includes predictions from the most commonly used prediction databases, and pathway analysis was conducted using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7) [538,539]. Statistical significance was determined as $P<0.05$.

Results

Exercise adherence

Of the 26 participants recruited into the intervention, 19 successfully completed the 12 exercise sessions. One participant withdrew due to a quadriceps musculoskeletal injury during training, one had a previous injury that was exacerbated by the exercise, another experienced an unrelated health concern and three stopped training for undisclosed reasons. The exercise adherence was approximately 73%.

Exercise improves cardiorespiratory health and fitness

Participants' phenotypes before and after exercise training are shown in Table 3. Exercise training increased cardiorespiratory fitness ($\dot{V}O_{2max}$) by 2.1ml·kg·min^{-1} (4.7%, *P*=0.03). Maximal running speed during $\dot{V}O_{2max}$ testing improved by 1km·hr^{-1} (4%, *P*=0.001) and LDL cholesterol was decreased after the intervention (-3.9%, *P*=0.047). Resting heart rate (-4%, *P*=0.05), diastolic BP (-4.3%, *P*=0.06) and total cholesterol (-3.3%, *P*=0.06) were reduced, while pulse pressure was increased (7.6%, *P*=0.07), all with borderline statistical significance.

Whole-genome leukocyte DNA methylation changes after exercise training

We analysed whole-genome DNA methylation using the 450 BeadChip (Illumina) to determine whether exercise altered the leukocyte methylome. The principle component analysis demonstrated separation between paired subject DNA methylation from samples taken before and after exercise training, indicating large changes to genome-wide DNA methylation (data not shown). DNA methylation changes occurred across the leukocyte genome in relation to gene regions and CpG islands (Figure 1). To gather an overview of where these DNA methylation changes had occurred, we determined the average DNA methylation before and after exercise in relation to CpG islands and location in relation to the

nearest gene. DNA methylation in relation to the nearest gene decreased in the promoter and intron regions for Infinium I and II assays, but increased in the 3' untranslated regions (UTR) for Inifinium I assay (Figure 2A, $P<0.0001$). There was a decrease in 5'UTR and exon DNA methylation in Infinium II assay (Figure 2A, $P<0.0001$). CpG island DNA methylation decreased in both Infinium I and II assays (Figure 2B, $P<0.0001$). Whereas Infinium II-assessed northern shore (N shore) DNA methylation deceased, southern shore (S shore) and northern shelf (N shelf) DNA methylation increased ($P<0.0001$, Figure 2B). Although the impact of specific regional DNA methylation changes are not completely understood, the CpG island and promoter region demethylation indicate an increase in transcriptional activity or gene un-silencing. Therefore, DNA methylation changes occurred across the leukocyte methylome after exercise training.

CpG methylation after exercise training

Out of the 485,577 CpG sites quantified for DNA methylation status on the Infinium HumanMethylation450 BeadChip (Illumina), exercise induced DNA methylation changes, ranging from 0.1–62.8%, at 205,987 sites relating to 32,445 transcripts after FDR ($q<0.05$). While 81,576 CpG sites relating to 16,256 transcripts became more methylated (Figure 2C, $q<0.05$), 124,411 CpG sites corresponding to 27,263 transcripts were de-methylated after exercise (Figure 2D, $q<0.05$).

In the search for biologically relevant changes to CpG site methylation, we identified CpG sites with a change of ≥5% after exercise ($q\leq0.05$) (Figure 3). Due to the large number of CpG sites (n=2,909) with a difference of ≥5%, we narrowed our focus to CpG sites (n=81) with a change of ≥10% ($q<0.005$, Table 4) and ≥20% after exercise ($q<0.005$, Table 5). Of the CpG sites that had large changes (≥20%) in DNA methylation after exercise, eight had increased methylation and 11 had less methylation after exercise. Next, after applying a stringent FDR ($q<0.001$) we used gene ontology to identify DNA methylation changes in

genes enriched for numerous cellular and molecular processes, including those involved in metabolic activity, biological adhesion and antioxidant activity (Figure 4). Using pathway analysis, we found that CpG sites altered by exercise (q<0.005) were those of genes related to pathways important for cardiovascular physiology, including focal adhesion, calcium signalling and MAPK signalling (q<0.05, Table 6). Therefore, differentially methylated CpG sites after exercise are enriched for pathways crucial for cardiovascular health.

DNA methylation validation

DNA methylation within a gene promoter region is typically associated with decreased gene expression, thus we focused on CpG sites with the largest and most statistically significant DNA methylation changes within gene promoter regions (≥10% and $P<1.0\times10^{-5}$). To that end, we analysed CpG sites within the *EGF* (cg12093976) and uracil-DNA glycosylase (*UNG)* (cg20982606) promoter regions using the EpiTYPER (Sequenom). We were unable to obtain meaningful data, due to the acquisition of peaks from additional fragments other than the fragment of interest.

Exercise-induced gene and miRNA expression

We then aimed to identify whether a change in DNA methylation caused by exercise would alter gene mRNA expression. We quantified the expression of genes that had a DNA methylation change (>10%) that were most statistically significant (q<0.001) within the promoter region or gene body (Figure 5A–F). We also quantified mature miR-21 and miR-210 as their genes had a modest (1.3–4.2%) change in DNA methylation at multiple regions across the gene (q<0.05, Table 7). These miRNAs are inversely correlated to cardiorespiratory fitness and implicated in cardiovascular disease [540].

Gene expression changes after the initial $\dot{V}O_{2max}$ test, at rest after exercise training and after the second $\dot{V}O_{2max}$ test (after exercise training) are displayed in Figure 5. We were unable to successfully amplify insulin-like family member 3 (*IGFL3*) mRNA, suggesting that this gene is lowly expressed in leukocytes. Genes fitted into one of five categories; specifically, 1) genes that were responsive after acute exercise at the beginning of exercise training only (cell division cycle 20, *CDC20*); 2) genes that responded to acute exercise before and after exercise training (isthmin 1, *ISM1*); 3) genes that responded to acute exercise before and after exercise training and that had altered gene expression at rest after exercise training (*EGF*, *UNG* and miR-210); 4) those that were only altered at rest after exercise training (miR-21); and finally, 5) those that were unchanged by acute or chronic exercise (chromobox homolog 7, *CBX7* and synuclein α interacting protein, *SNCAIP*, data not shown). Interestingly, the demethylated (11.6% and 12.9%, respectively) *EGF* and *UNG* promoter was accompanied by a significant decrease and increase in mRNA expression, respectively (relative expression ± SEM, 10.75±1.68 to 8.25±0.95, $P<0.05$ and 1.72±0.14 to 2.12±0.15, $P=0.05$). These data suggest exercise training-induced DNA methylation changes within genes (including miRNA genes) cause changes to mRNA and mature miRNA levels (Figure 6).

miRNA-mRNA targets

Finally, we used miRWalk,[537] to predict mRNA targets of miR-21 and miR-210. We then used DAVID [538,539] to identify pathways and diseases associated with mRNA molecules targeted by miR-21 and miR-210. Notably, miR-21 mRNA targets were enriched for pathways including MAPK signalling, Toll-like receptor signalling, apoptosis and fatty acid metabolism. Moreover, mRNA targets of miR-210 were enriched for genes relating to calcium signalling, B-cell receptor signalling and TGF-beta signalling pathways. While the mRNA targets of miR-21 were from genes associated with diseases, such as ischemic heart disease (n=6), nephropathy (n=5) and coronary atherosclerosis (n=11), miR-210 mRNA

targets were from genes associated with focal dystonia (n=2). Collectively, these data suggest a role for miR-21 and miR-210 in the positive cardiovascular adaptations associated with short-term intense exercise training.

Discussion

Our study is the first, to our knowledge, to show genome-wide leukocyte DNA methylation changes caused by short-term exercise in healthy young men. We found global and specific leukocyte DNA methylation changes with concomitant changes to mRNA and miRNA expression, in addition to favourable cardiovascular health adaptations after four weeks of exercise training. Specifically, we observed DNA methylation changes to CpG islands and gene promoter and gene bodies. We showed exercise training-induced demethylation of cg12093976 and cg20982606, which decreased and increased the mRNA expression of the *EGF* and *UNG* genes, respectively. Furthermore, exercise training-induced leukocyte DNA methylation changes across miRNA genes (*MIR21* and *MIR210*) known to influence cardiovascular physiology [541,542], subsequently influencing their mature miRNA expression.

Sprint interval training causes cardiopulmonary and metabolic adaptations similar to that of traditional long duration aerobic training, but demands dramatically less time commitment [543]. Given a common barrier to engagement in exercise is lack of time, sprint interval training may be an attractive alternative exercise training regime that improves health and fitness. We demonstrated that four weeks of sprint interval training increased $\dot{V}O_{2max}$ and reduced total and LDL cholesterol in healthy young men, thereby improving cardiorespiratory fitness and blood lipid profile. We are, to our knowledge, the first to demonstrate that four weeks of sprint interval training can significantly decrease LDL cholesterol in young men. Although some have demonstrated eight weeks of sprint interval training, in the form of running, is

effective at reducing LDL and total cholesterol [544], other shorter (six week) sprint interval training interventions have not reduced LDL cholesterol [545]. Considering the role of LDL cholesterol in blood vessel health [546,547], sprint interval training may be a cheap and effective short-term strategy to promote a favourable blood lipid profile.

Global leukocyte DNA methylation changes seem to be influenced by physical activity and exercise training but data is equivocal. Although global leukocyte DNA methylation is increased in middle-aged individuals performing moderate [548] or high amounts [549] of physical activity in some studies, in others, global methylation is inversely [550,551] related to physical activity or unrelated [552]. The DNA methylation of exon 1 of the *PYCARD* gene was increased in older adults who engaged in six months of moderate intensity exercise, to levels comparable to that of their younger peers [524]. We are the first to analyse the exercise training-induced changes to genome-wide leukocyte DNA methylation in healthy subjects. Notably, we found a subtle decrease in leukocyte global methylation, consistent with previous findings [550,551]. These data may indicate wide-spread transcriptional changes to leukocytes. In particular, we found CpG island and gene body and promoter regions were, on average, demethylated after exercise training, indicating the modulation of transcriptional activity.

The exercise-induced changes to skeletal myocyte [522] and adipocyte [523] genome-wide DNA methylation have been analysed previously. Strikingly, the exercise training-induced changes to DNA methylation found in our study included CpG sites in genes involved in pathways similar to that previously shown to be affected in skeletal myocytes after six months of exercise training [522]. For example, six months of moderate intensity exercise altered skeletal muscle DNA methylation in genes related to MAPK signalling, progesterone-mediated oocyte maturation, Wnt signalling, Melanogenesis, hedgehog signalling and calcium signalling pathway [522] – pathways also modulated in leukocytes of individuals from our study (Table 6). Furthermore, some of the mentioned pathways regulated by DNA methylation

changes caused by exercise may also be governed by miRNA-mediated mRNA regulation, as indicated by our miRNA pathway analysis.

Whereas skeletal myocytes and adipocyte DNA methylation changes are predominantly indicative of metabolic transcriptional changes caused by exercise, leukocytes changes could be used as biomarkers of systemic changes to health and fitness. Leukocytes circulate through the body and their gene expression mimics that of their internal and external environments, making them useful biomarkers [25]. Likewise, the epigenetic landscapes are malleable to the internal (biological) and external environments (such as physical exercise) [553,554]. Therefore, leukocyte changes to epigenetic modifications could be relevant biomarkers of phenotype changes and in this case, biomarkers of the changes in cardiorespiratory fitness and accompanying health and performance adaptations (total and LDL cholesterol, and maximal running speed). Leukocytes are also implicated in the pathogenesis of atherosclerosis [525] and the DNA methylation changes observed in our study provide mechanistic insights into how exercise attenuates the risk of atherosclerosis.

Interestingly, exercise training caused DNA methylation changes with paralleled miRNA changes. DNA methylation can down-regulate gene expression by working in concert with other epigenetic modifications (e.g. Histone protein methylation and acetylation) and DNA binding proteins to compact chromatin and, in turn, inhibit transcription factor binding [44,555-557]. We identified that DNA methylation changes to *MIR21* and *MIR210* genes influenced the mature miR-21 and miR-210 expression. The microRNAs miR-21 and miR-210 are ubiquitously expressed hypoxamirs involved in cardiovascular diseases, inflammation and angiogenesis [558,559]. Indeed, miR-21 is up-regulated in atherosclerotic

plaques [560] and acute coronary syndromes [561]. The increased miR-21 expression, however, may be a protective response to the disease and acute coronary damage. For example, up-regulated miR-21 was protective against ischemia-induced cell apoptosis by down-regulating programmed cell death 4 (*PDCD4*) mRNA after myocardial infarction in rats [562]. Previous *in vitro* experiments showed miR-21 attenuates vascular smooth muscle cell apoptosis caused by reactive oxygen species (hydrogen peroxide, H_2O_2) through down-regulation of programmed cell death 4 (*PDCD4*) [563]. Additionally, human endothelial cell miR-21 was augmented by shear stress and lead to decreased phosphatase and tensin homolog (*PTEN*)-mediated apoptosis, facilitated endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide (NO) production [564]; an effect exerted by exercise training [565,566]. miR-21 is particularly responsive to exercise training as it is up-regulated after chronic exercise training in serum [567], spinal cord [568], cardiac [569] and skeletal myocytes [570] in mammals. Thus, exercise may maintain vascular integrity and prevent vascular disease through up-regulating miR-21 expression. This, in turn, contributes to modulation of miR-21 downstream mRNA targets involved in the eNOS pathway, oxidative stress and apoptosis.

miR-210 is induced by hypoxia and is up-regulated in numerous cardiovascular diseases, especially those associated with ischemia [542]. Similar to miR-21, miR-210 is up-regulated in atherosclerotic plaques [560] and myocardial infarction [571], possibly by oxidative damage and cellular senescence. Reactive oxygen species (ROS) up-regulates miR-210 through phosphorylation of AKT, ERK1/2 and platelet-derived growth factor receptor β [572]. miR-210 is also known to facilitate ROS production and to induce double-stranded DNA breaks in human cells undergoing replicative senescent [573]. Interestingly, serum miR-21 and -210 were shown to be inversely correlated to $\dot{V}O_{2max}$ in a cohort of 100 healthy subjects [540]. Our data

also suggests miR-210 may be indicative of $\dot{V}O_{2max}$, as our participants exhibited elevated $\dot{V}O_{2max}$ with decreased leukocyte miR-210 expression after exercise training. We showed predicted targets of miR-21 are particularly enriched for mRNA from genes associated with cardiovascular pathology (ischemic heart disease and coronary atherosclerosis) that supports the concept but warrants further study. The impact of these exercise-induced miRNA changes on genes and biological implications is also left for future research. Collectively, it is likely that miRNAs, miR-21 and miR-210, are exercise-responsive miRNAs that underpin the salubrious adaptations associated with exercise training.

Demethylation of *EGF* and *UNG* gene promoter regions attenuated and augmented the mRNA levels, respectively. Promoter demethylation is generally associated with gene activation [44], but not exclusively, as promoter demethylation does not always increase gene expression [574-576]. Alternatively, the observed decrease to *EGF* mRNA expression could be due to other means of transcriptional regulation such as histone modifications, small non-coding RNA molecules or transcription factor activity. Importantly, EGF is a ligand for the epidermal growth factor-receptor (EGFR), which is hyperactive in atherosclerosis [577]. The increased EGFR activity by elevated EGF may facilitate increased inflammation and blood vessel damage, leading to atherosclerosis [577]. Acute exercise training increases peripheral but not central artery distensibility [528], which is an effect established after chronic exercise training [527]. Moreover, exercise training increases and decreases muscle microvascular density and arterial stiffness, respectively [578]. Therefore, the present study indicates that the attenuation of the EGFR-ligand, *EGF*, expression by promoter DNA methylation changes caused by exercise training could benefit vascular health. Similarly, the observed DNA methylation and gene expression change of *UNG* could serve as a mechanism for the demethylation of most genes analysed in the present study. Active DNA demethylation occurs via the hydroxylation of 5-methylcytosine to 5-hydroxymethylcytosine by ten-eleven

translocation (TET) proteins (1-3), followed by the active base-excision repair initiated by UNG [579,580]. Together, these data demonstrate the complex regulation of gene expression; where DNA methylation can directly regulate mRNA levels or, indirectly modulate gene expression through the modulation of miRNA expression subsequently influencing gene expression and cardiovascular phenotypes.

Although not measured, the numerous changes to DNA methylation observed in the present study could be a result of the altered DNA methyltransferase enzymes (DNMT1, 3A and 3B). While DNMT1 is responsible for maintaining DNA methylation during mitosis, DNMT3A and -3B are responsible for *de novo* methylation changes [581]. It is possible that exercise training-induced changes to activity of DNMT enzymes could have impacted the leukocyte methylome as there expression was altered in the hippocampi of mice after seven days of voluntary wheel running [582]. Passive loss of DNA methylation during cell division is an alternate explanation for the DNA methylation changes observed in our study. This is, however, unlikely because of the short duration of study intervention period. The DNA methylation changes (up to 62.8%) caused by exercise training in our study could be explained by the intensity of exercise training. Previous work [526] revealed skeletal myocyte global and metabolic gene (*PPARGC1A*, *TFAM*, *PPARD*, *MEF2A*) DNA demethylation is intensity dependent, where higher intensity exercise elicited greater DNA demethylation. Moreover, the acute increase in reactive oxygen species and other metabolites may influence TET proteins and histone acetyltransferase and deacetylase activity to alter DNA methylation [583], but this remains to experimentally demonstrated.

Our study has some limitations. Blood leukocyte counts were not quantified before and after exercise training and it is possible that shifts in leukocyte subsets could be responsible for the change in leukocyte DNA methylation observed in our study. This is, however, unlikely as we quantified leukocyte DNA methylation from resting blood samples unaffected by the

leukocytosis associated with acute exercise. Furthermore, there is no evidence to suggest leukocyte subsets change with chronic exercise training and a similar high-intensity exercise training study did not alter leukocyte subsets [584]. Whist we did not include any experimental controls (who did not participate in any exercise for four weeks), data from our laboratory indicates that genome-wide leukocyte DNA methylation is stable over the course of eight weeks, as no changes were observed in a cohort of eight young men not engaging in any high-intensity aerobic exercise (unpublished data). We could not successfully validate the 450K DNA methylation results using EpiTYPER due to issues with the genomic regions being analysed. Exercise training was not performed in a laboratory setting and participants exercise intensity was not directly monitored during the course of the intervention. Lastly, we cannot rule-out potential changes to participants' diet caused DNA methylation changes.

In conclusion, we have demonstrated that four weeks (249 min) of exercise training significantly alters the leukocyte methylome. These DNA methylation changes influenced miRNA and mRNA levels, improved cardiorespiratory fitness and enhanced the blood lipid profile of healthy, young men. These DNA methylation changes could be relevant biomarkers for monitoring adherence to exercise interventions and demonstrates a dynamic role for epigenetic regulation in the change to cardiovascular health and fitness caused by short-term exercise training.

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Disclosures

None.

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Figure Captions

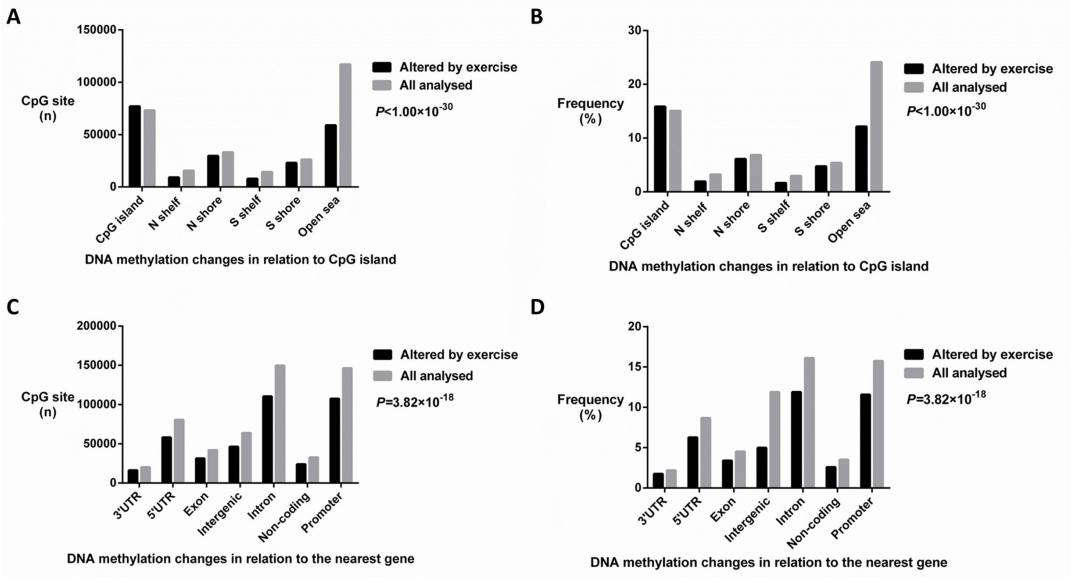

Figure 1 DNA methylation changes in relation to CpG islands (A and B) and the nearest gene (C and D), respectively. Data are from Chi^2 tests.

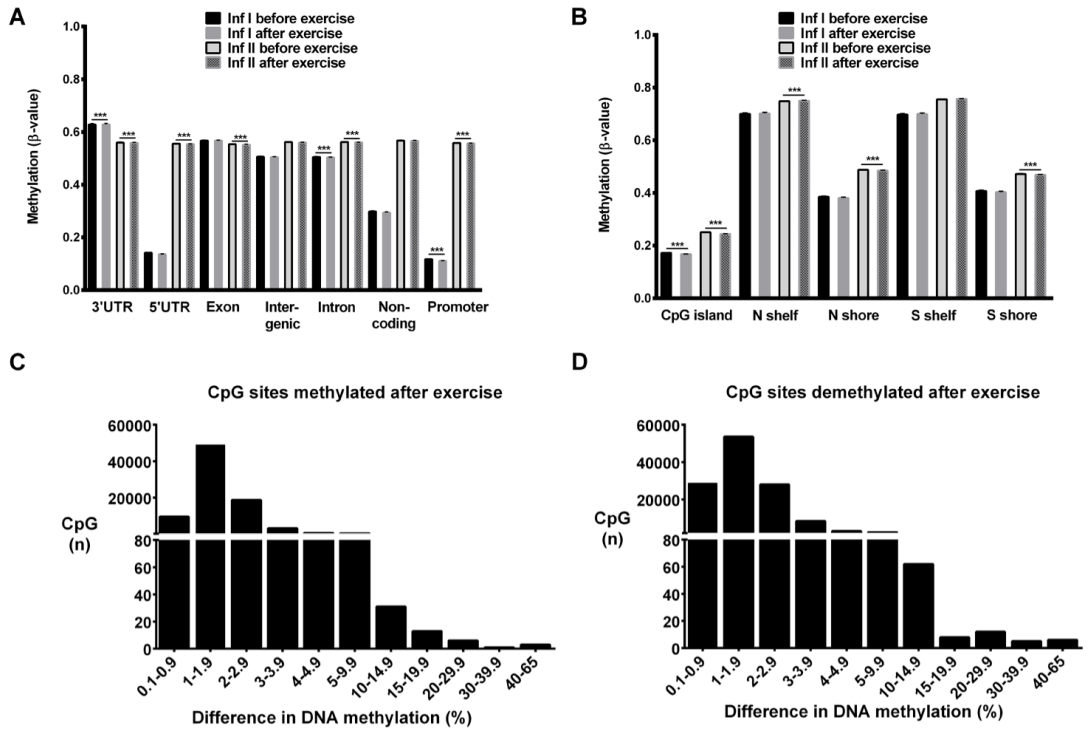

Figure 2. Whole-genome DNA methylation changes caused by exercise. Genome wide DNA methylation across all 485,577 CpG sites analysed on the Infinium HumanMethylation450 BeadChip (Illumina, Australia) were compared before and after exercise in relation to A) nearest gene and B) CpG island. Data are from Wilcoxon matched-pair signed ranked test and are expressed as mean ± SEM. Number of CpG sites with C) increased methylation and D) decreased methylation, with magnitude of DNA methylation change after exercise (q<0.05).

Legend: ***P<0.0001; N: northern; S: southern; 3'UTR: 3-prime un-translated region; 5'UTR: 5-prime un-translated region; Promoter: gene promoter region (1–1500 bases upstream of the transcription start site).

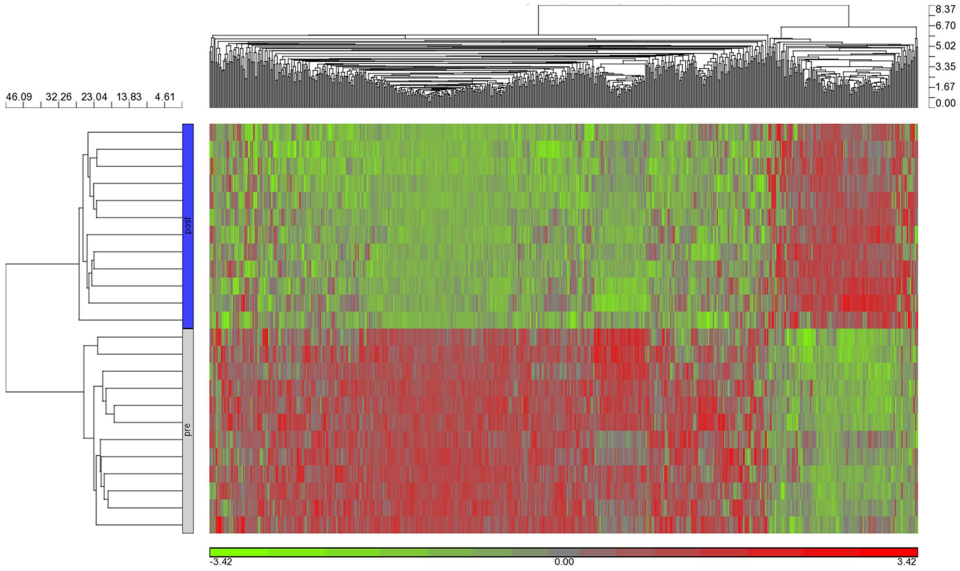

Figure 3. Hierarchical clustering of CpG methylation before and after exercise training.

The CpG methylation status is indicated by green (low methylation) and red (high methylation) in 12 subjects pre (grey bar) and post (blue bar) exercise training. Each branch of the array tree on the left of the grey and blue bars are subjects and are separated before and after the exercise training. The array tree at the top of the hierarchical cluster indicates clusters of CpG sites modified by exercise.

Data are from CpG sites with a fold-change of >5% (q<0.001).

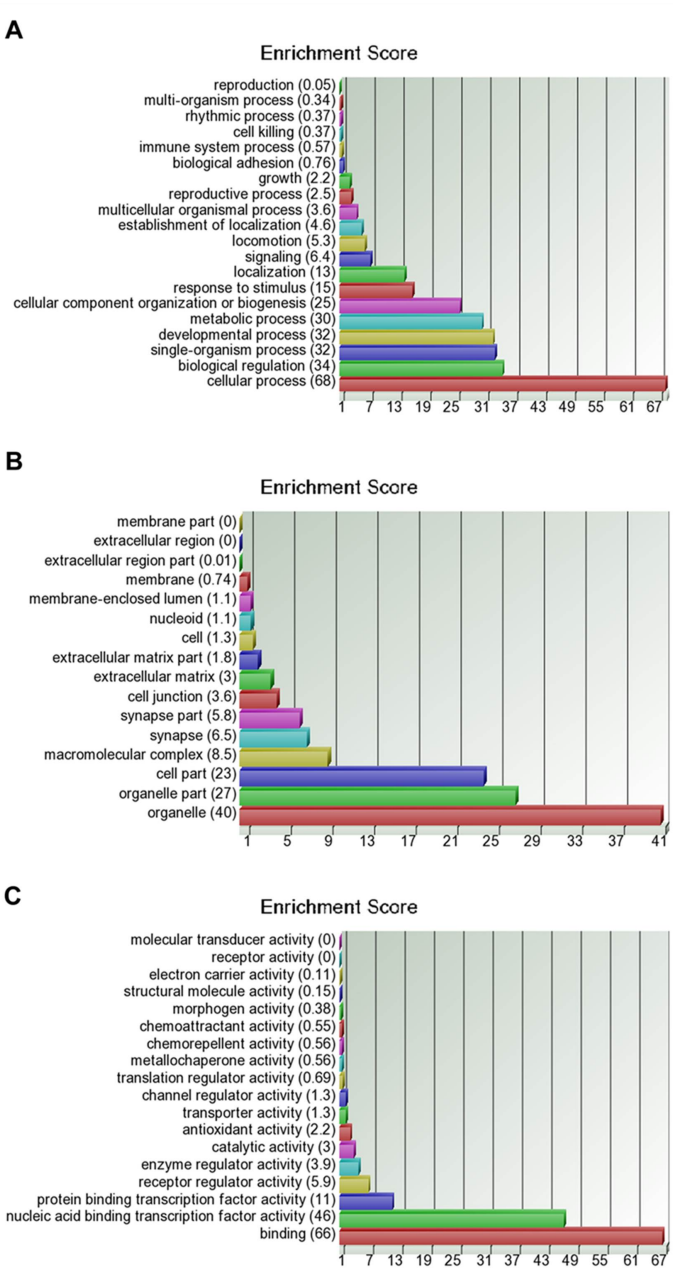

Figure 4. Gene ontology for genes with CpG DNA methylation changes after exercise. Gene ontology is based on CpG sites with altered DNA methylation (q<0.001) after exercise training. Gene ontology enrichment scores are given and organised by A) biological processes; B) cellular components; and C) molecular functions.

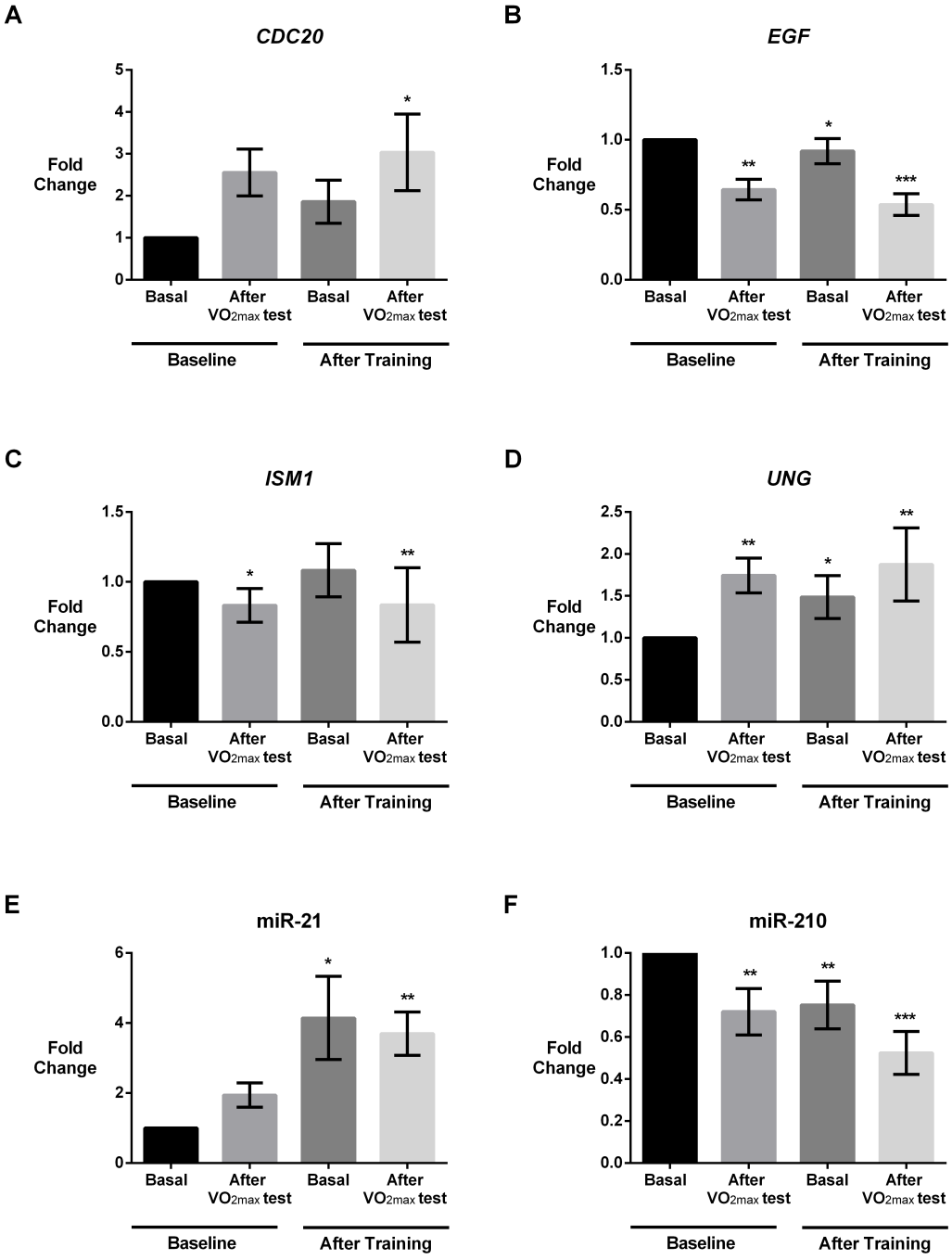

Figure 5. Gene and miRNA expression changes caused by exercise. Data are from two-way repeated-measures ANOVA and are expressed as fold change of genes and mature miRNA expression (mean ± SEM). A significant difference between basal and other time-points is indicated by asterisks.

Legend: **P*<0.05; ***P*<0.01; ****P*<0.001 vs basal.

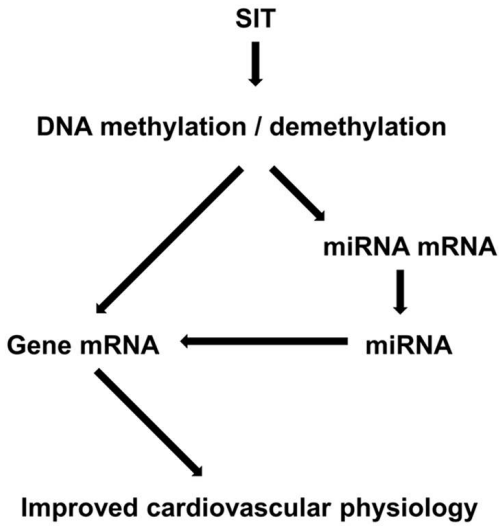

Figure 6. Schematic of DNA methylation regulation on cardiovascular physiology.

Exercise training, in the form of sprint interval training (SIT), influences the leukocyte DNA methylome. This, in turn, modulates the expression (mRNA levels) of protein-coding genes (*EGF* and *UNG*) and also microRNAs (miR-21 and -210). MicroRNAs, miR-21 and miR-210 would subsequently regulate other protein-coding genes to inevitably influence cardiovascular physiology and enhance cardiovascular health and performance.

Tables

Table 1. Description of the four week exercise program.

Week	Session #	Training load*	Training sprint time (min)	Total session time (min)
1	1	3 sprints	1.3	9.5
	2	4 sprints	2	14
	3	5 sprints	2.5	18.5
2	4	4 sprints	2	14
	5	5 sprints	2.5	18.5
	6	6 sprints	3	23
3	7	5 sprints	2.5	18.5
	8	6 sprints	3	23
	9	7 sprints	3.5	27.5
4	10	6 sprints	3	23
	11	7 sprints	3.5	27.5
	12	8 sprints	4	32
	Total Time	66	33	249

Legend: # number; * all sprints were completed at maximum intensity and separated by four minutes of passive recovery.

Table 2. TaqMan Assay information.

Gene/microRNA	**TaqMan ID**
CBX7	Hs00545603_m1
CDC20	Hs00426680_mH
EGF	Hs01099999_m1
GAPDH (mRNA reference gene)	Hs02786624_g1
IGFL3	Hs00419511_g1
ISM1	Hs01382748_m1
SNCAIP	Hs00917423_m1
UNG	Hs01037093_m1
hsa-miR-21	000397
hsa-miR-210	000512
RNU44 (miRNA reference gene)	001094

Table 3. Participant phenotypes before and after exercise training.

Phenotype	Before exercise Mean±SD	After exercise Mean±SD	% Change	*P*-value
		n=19		
Age (years)		21.1±2.7		
Height (cm)		180.6±7.4		
Anthropometric				
Weight (kg)	78.1±9.4	78.1±9.8	-0.03±2.3	0.99
BMI (kg/m^2)	23.9±2.3	23.9±2.4	-0.2±2.4	0.68
Waist (cm)	79.2±5.3	78.9±4.8	-0.4±2.5	0.50
Hip (cm)	82.8±6.0	82.4±5.4	-0.3±1.8	0.37
WHR	0.96±0.02	0.96±0.02	-0.1±2.3	0.94
∑ 7 skinfolds (cm)	98.1±38.3	91.3±30.7	-4.5±13.2	0.13
Body density	1.07±0.01	1.07±0.01	0.2±0.5	0.13
Fat %	13.0±4.9	12.2±4.1	-4.2±12.7	0.11
Cardiovascular				
RHR (beats/min)	64.1±10.8	59.5±9.3	-4.0±18.2	0.055
SBP (mmHg)	124.0±12.1	124.8±7.8	1.1±10.5	0.77
DBP (mmHg)	72.0±6.7	69.5±7.0	-3.4±7.9	0.06
MAP (mmHg)	89.4±7.9	87.9±6.7	-1.3±8.8	0.42
PP (mmHg)	51.9±8.4	55.4±5.8	7.6±16.8	0.07
Total CHOL (mmol/L)	4.42±0.79	4.24±0.67	-3.3±9.3	0.06
Triglycerides (mmol/L)	0.97±0.35	0.95±0.48	-4.2±22.4	0.70
HDL-C (mmol/L)	1.34±0.24	1.31±0.20	-0.9±11.7	0.50
LDL-C (mmol/L)	2.64±0.78	2.48±0.61	-3.9±11.2	**0.047**
LDL-C/HDL-C (mmol/L)	2.06(1.5–2.8)	2.0(1.5–2.5)	-1.6±15.0	0.24

CHOL/HDL-C (mmol/L)	3.41(2.7–4.2)	3.31(2.7–4.2)	-2.0±9.1	0.35
Cardiorespiratory fitness				
VO_{2max} ($ml \cdot kg \cdot min^{-1}$)	49.5±4.7	51.6±3.9	4.7±10.1	**0.03**
Final treadmill Speed ($km \cdot hr^{-1}$)	19(18–21)	20(19–21)	4.0±5.1	**0.001**

Data are expressed as mean ± standard deviation or as mean (interquartile range) from paired t-tests or related samples Wilcoxon signed rank test, respectively.

Legend: ∑, sum of; % change, percentage change; BMI, Body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; PP, pulse pressure; CHOL, cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 4. DNA methylation changes after four weeks of exercise training (≥10%, $q \leq 0.005$).

Location in relation to					***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg19933985			S shelf	5	53.8±4.4	70.8±4.1	17	1.93×10^{-7}	1.49×10^{-5}
cg10633981	*C11orf58*	3'UTR	Open sea	11	49.5±6.7	66.4±5.1	16.9	2.13×10^{-8}	3.97×10^{-6}
cg02732134	*DNMBP*	5'UTR	Open sea	10	42.3±2.8	57.9±3.6	15.6	4.79×10^{-8}	6.26×10^{-6}
cg01287788	*SNORD94* *PTCD3*	TSS200 Body	Open sea	2	49.5±4.2	65.0±5.2	15.5	2.96×10^{-7}	1.96×10^{-5}
cg27379715			Open sea	2	55.1±3.3	70.0±3.3	14.8	8.39×10^{-9}	2.39×10^{-6}

cg17393016	*C17orf55*	TSS1500	N shore	17	54.2±13.2	68.7±15.9	14.5	0.002	0.005
cg02308712			Open sea	12	47.5±3.9	61.7±4.3	14.1	9.38×10^{-8}	9.45×10^{-6}
cg15897635			N shelf	1	30.7±6.6	44.4±5.3	13.8	0.0003	0.001
cg21937244	*CDC42BPB*	Body	Island	14	64.4±5.9	78.0±3.4	13.7	3.10×10^{-8}	4.86×10^{-6}
cg12578536			S Shelf	5	48.44.0	61.8±3.5	13.4	2.74×10^{-7}	1.86×10^{-5}
cg17931986	*COL11A2*	3'UTR	S shore	6	53.8±3.1	67.2±3.6	13.4	6.86×10^{-9}	2.16×10^{-6}
cg04205664	*CLEC2L*	Body	S shore	7	28.3±1.9	41.5±4.1	13.2	2.09×10^{-7}	1.57×10^{-5}
cg05239225	*AKAP13*	5'UTR	Open sea	15	46.6±7.3	59.5±4.3	12.9	0.0003	0.001
cg13810766	*PRKAG2*	Body	Open sea	7	33.6±1.9	45.9±3.1	12.3	2.72×10^{-9}	1.39×10^{-6}
cg12949141	*PCBD2*	Body	Open sea	5	35.1±5.6	47.1±4.9	12.1	8.56×10^{-5}	0.0007
cg02771392	*ZNF273*	TSS1500	Open sea	7	59.3±3.1	71.3±2.0	12	1.58×10^{-8}	3.35×10^{-6}

cg04250181	*SLCO1B3*	5'UTR	Open sea	12	65.0±6.0	76.9±3.5	11.9	1.90×10^{-6}	6.24×10^{-5}
cg01775802	*RGS6*	Body	Open sea	14	27.6±5.1	39.3±5.2	11.7	9.61×10^{-5}	0.0007
cg22870994			Open sea	11	44.3±2.3	55.9±2.4	11.6	1.06×10^{-7}	1.02×10^{-5}
cg14497545	*MAML3*	Body	Open sea	4	36.5±2.3	48.1±4.0	11.5	5.62×10^{-7}	2.95×10^{-5}
cg23817637	*CLRN3*	TSS1500	Open sea	10	60.3±6.2	71.8±2.6	11.5	0.0001	0.0009
cg18022036			Open sea	5	47.3±3.9	37.4±4.6	-10	0.0001	0.001
cg06611444			Open sea	4	70.1±5.5	60.2±5.4	-10	8.78×10^{-6}	0.0002
cg16859420			Open sea	8	31.6±5.4	21.6±3.9	-10	0.0002	0.001
cg20090957	*MAPKAPK5* *C12orf47*	TSS1500 Body	Island	12	34.1±3.1	24.1±2.4	-10	2.10×10^{-6}	6.63×10^{-5}
cg17502267	*BNIP2*	Body	Island	15	39.4±2.5	29.4±4.0	-10	2.59×10^{-5}	0.0003

cg22876894			*N shore	20	31.5±4.2	21.5±2.6	-10	1.25×10^{-7}	1.13×10^{-5}
cg18825597			N shelf	8	61.2±4.4	51.1±6.4	-10.1	0.001	0.004
cg16146432	*C3orf50*	TSS200	N shore	3	26.1±4.5	16.1±3.1	-10.1	1.52×10^{-6}	5.43×10^{-5}
cg17479131	*LOC401431*	Body	N shelf	7	54.2±2.3	44.1±4.4	-10.1	5.14×10^{-5}	0.0005
cg06180061	*C16orf91*	Body	S shelf	16	62.2±3.5	52.0±4.6	10.1	1.98×10^{-8}	3.84×10^{-6}
cg08339189	*GGTA1*	Body	N shore	9	21.8±1.7	11.6±2.3	10.2	1.71×10^{-6}	5.83×10^{-5}
cg17932934	*CALN1*	5'UTR 1st Exon	Open sea	7	72.9±2.1	62.7±5.7	10.2	3.92×10^{-5}	0.0004
cg14366742			Open sea	4	43.4±4.0	33.2±4.6	10.2	5.62×10^{-8}	6.85×10^{-6}
cg11381792	*TRAPPC10*	Body	Island	21	31.9±1.3	21.7±1.8	10.2	1.64×10^{-9}	1.12×10^{-6}
cg04669668	*BANP*	Body	Island	16	57.2±3.7	46.9±4.8	10.3	0.0001	0.0008

cg09700085	*SLC6A20*	5'UTR 1st Exon	Island	3	26.0±2.2	15.7±1.9	10.3	2.08×10^{-8}	3.93×10^{-6}
cg08206623	*CDKN1C*	TSS1500	Island	11	45.8±1.5	35.5±1.8	10.3	2.61×10^{-8}	4.38×10^{-6}
cg20144008			Island	2	45.3±2.1	35.0±2.4	10.4	1.11×10^{-8}	9.05×10^{-7}
cg07894334	*SOCS5*	TSS1500	†N shore	2	59.2±7.2	48.8±5.8	10.5	2.78×10^{-5}	0.0003
cg27130012			S shore	6	74.6±2.7	64.1±4.6	10.5	1.02×10^{-5}	0.0002
cg15386880			Island	16	50.5±4.8	40.0±7.5	10.5	0.0001	0.0009
cg00001793	*ETV6*	Body	Open sea	12	59.2±5.0	48.7±7.1	10.5	0.0001	0.0008
cg21265548	*NSDHL* *CETN2*	5'UTR TSS1500	‡S shore	X	46.3±8.1	35.7±7.1	10.6	0.001	0.004
cg05280527	*NRXN3*	3'UTR	S shore	14	47.7±3.0	37.1±3.5	10.6	9.17×10^{-8}	9.35×10^{-6}

cg02678768	*EVPL*	3'UTR	N shore	17	67.1±9.1	56.4±8.7	10.7	3.77×10^{-6}	9.51×10^{-5}
cg23917513	*LMX1A*	5'UTR Body	Open sea	1	66.1±4.1	55.4±5.5	10.7	3.92×10^{-5}	0.0004
cg13985765	*PCSK6*	Body	Open sea	15	68.7±6.4	57.9±6.4	10.8	0.0005	0.002
cg25152348	*NCAPH2* *LMF2*	5'UTR 1^{st} Exon TSS1500	Island	22	47.2±2.1	36.3±1.8	10.9	5.29×10^{-8}	6.62×10^{-6}
cg16500605	*BAT3*	TSS1500	Island	6	31.9±3.5	20.9±1.4	11	1.05×10^{-6}	4.29×10^{-5}
cg01558909	*HBM*	TSS200	Island	16	14.8±9.0	3.8±0.5	11	0.0006	0.002
cg15694789	*STARD8*	5'UTR	S shore	X	38.3±4.6	27.3±3.3	11	2.69×10^{-6}	7.71×10^{-5}
cg05524038	*CSF1R*	TSS1500	Open sea	5	80.0±2.9	68.9±2.5	11.1	1.51×10^{-6}	5.42×10^{-5}

cg11123847	*ACTR10*	3'UTR	Open sea	14	80.9±3.1	69.8±4.3	11.2	1.14×10^{-5}	0.0002
cg14506366	*SLC6A3*	Body	Open sea	5	66.5±7.0	55.3±6.6	11.2	0.001	0.003
cg06082548	*NKX6-2*	Body	*Island	12	32.3±2.1	21.0±1.9	11.3	3.50×10^{-10}	5.28×10^{-7}
cg26979339	*RIC8B*	TSS1500	Island	12	35.7±1.5	24.3±2.2	11.4	1.45×10^{-8}	3.23×10^{-6}
cg25474648	*ZDHHC14*	3'UTR	S shore	6	74.2±2.5	62.7±4.8	11.5	2.62×10^{-6}	7.58×10^{-5}
cg01994308	*PLAG1* *CHCHD7*	5'UTR TSS1500	N shore	8	40.0±6.7	28.5±4.2	11.5	0.0005	0.002
cg12093976	*EGF*	TSS200	Open sea	4	34.3±2.5	22.8±3.0	11.6	7.18×10^{-9}	2.20×10^{-6}
cg12861974	*CXorf57*	TSS200	Island	X	33.4±4.8	21.8±2.9	11.6	3.04×10^{-6}	8.32×10^{-5}
cg20929922	*C5orf28*	TSS1500	S shore	5	37.7±3.1	26.0±3.4	11.7	3.23×10^{-5}	0.0004
cg19349861	*OPCML*	5'UTR 1st Exon	Open sea	11	34.7±3.0	22.9±2.3	11.7	1.62×10^{-8}	3.41×10^{-6}

cg03909902	*PRDM4*	5'UTR	Island	12	35.6±2.8	23.8±1.7	11.8	1.75×10^{-8}	3.54×10^{-6}
cg04981696	*ABCC1*	Body	S shore	16	41.5±4.3	29.5±4.0	12	3.30×10^{-6}	8.76×10^{-5}
cg05005382	*CHTF18*	Body	Island	16	68.5±3.3	56.4±5.0	12.1	0.0003	0.001
cg00556029	*MARCKSL1*	TSS200	Island	1	30.9±4.8	18.7±2.6	12.2	3.22×10^{-5}	0.0004
cg03252499			Open sea	11	63.3±7.4	51.0±5.3	12.3	0.001	0.004
cg25501666			N shelf	2	65.8±4.5	53.1±4.8	12.7	3.68×10^{-6}	9.35×10^{-5}
cg20982606	*UNG*	TSS1500 TSS200	Island	12	29.1±3.0	16.2±2.4	12.9	9.23×10^{-9}	2.52×10^{-6}
cg16379462	*SAMD4A*	Body	Open sea	14	64.6±3.8	51.6±6.4	12.9	0.0004	0.002
cg14266237	*KLF1*	3'UTR	N shore	19	49.8±6.9	36.8±8.3	13	3.93×10^{-8}	5.62×10^{-6}
cg21393587			N shore	X	74.4±2.4	61.4±4.0	13	5.53×10^{-8}	6.76×10^{-6}
cg06808467	*LOC339290*	TSS1500	Island	18	39.2±2.4	25.6±3.1	13.6	2.56×10^{-8}	4.36×10^{-6}

	C18orf18	Body							
	DALRD3	5'UTR							
	NDUFAF3	TSS1500							
cg17092349			N shore	3	47.5±6.6	33.8±5.8	13.7	5.10×10^{-5}	0.0005
	MIR425	TSS1500							
	MIR191	TSS200							
cg26081875			Open sea	6	36.2±7.9	22.3±5.3	13.9	0.001	0.004
cg01525244	*CBX7*	TSS200	N shore	22	30.3±1.2	16.3±1.9	14	4.20×10^{-10}	5.96×10^{-7}
		5'UTR							
cg09844907	*MPV17L*		Island	16	34.0±1.5	19.4±2.6	14.6	1.87×10^{-8}	3.70×10^{-6}
cg27494111			N shore	19	67.6±5.1	52.5±10.4	15.1	3.25×10^{-6}	8.68×10^{-5}
cg26186549			S shore	6	68.7±11.4	53.3±11.9	15.4	2.77×10^{-5}	0.0003
cg16524049	*LMX1A*	Body	Island	1	43.1±2.7	27.1±4.6	16	4.54×10^{-6}	0.0001

Data are expressed are beta values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site; *DMR, differentially methylated region; †CDMR, cancer differentially methylated region; ‡RDMR, reprogrammed differentially methylated region.

Table 5. DNA methylation changes after four weeks of exercise training (≥20%, $q \leq 0.005$).

	Location in relation to				***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg21036194	*SNCAIP*	Body	Open sea	5	24.1±23.1	86.2±1.8	62.1	5.05×10^{-7}	2.76×10^{-5}
cg22588144	*ISM1*	TSS1500	Island	20	4.9±1.0	53.1±8.3	48.2	1.69×10^{-12}	5.35×10^{-8}
cg01309395	*HLA-DPB2*	Body	Open sea	6	37.9±34.7	82.1±4.1	44.2	0.0004	0.002
cg07658590	*SLC19A1*	TSS1500	S shore	21	15.6±18.2	47.6±3.9	32.0	0.0004	0.002
cg18531559	*ULK4*	TSS200	S shore	3	8.4±1.9	36.1±0.6	27.7	1.70×10^{-11}	1.56×10^{-7}
cg11950805	*CDC20*	TSS1500	Island	1	1.7±0.5	25.8±15.0	24.1	5.47×10^{-5}	0.0005
cg26919805	*PPPDE2*	5'UTR 1st Exon	Island	22	1.9±0.5	25.2±18.2	23.3	0.001	0.003
cg09459740	*XRCC6*	TSS1500	Open sea	11	74.1±1.2	96.2±5.2	22.1	9.12×10^{-8}	9.34×10^{-6}
cg07170824	*ACVRL1*	5'UTR 1st Exon	Island	12	52.7±3.1	32.7±5.3	-20.0	2.51×10^{-7}	1.75×10^{-5}
cg16700025	*CMIP*	Body	Open sea	16	62.2±15.4	36.1±0.6	-26.0	0.0001	0.001
cg18684755	*RYR1*	Body	S shelf	19	86.9±1.6	56.7±25.4	-30.2	0.002	0.005

cg06390613	*TMEM198*	3'UTR	N shelf	2	76.2±2.0	43.9±18.2	-32.3	0.0001	0.0009
cg03229033			Island	1	44.5±5.8	12.0±21.6	-32.4	0.0001	0.0009
cg15224432	*IGFL3*	TSS1500	Open sea	19	73.8±1.8	36.1±0.6	-37.7	7.17×10^{-16}	4.55×10^{-11}
cg04600795			Open sea	6	88.7±1.2	45.1±20.9	-43.7	1.50×10^{-5}	0.0002
cg10580110	*KRT6A*	TSS1500	Open sea	12	88.5±1.7	44.8±29.6	-43.7	1.40×10^{-5}	0.0002
cg08975528	*ZBTB12*	3'UTR	Island	6	74.6±2.9	19.8±34.1	-54.7	0.0002	0.001
cg17415355	*TOLLIP*	Body	S shelf	11	86.5±1.5	24.9±35.8	-61.6	8.60×10^{-5}	0.0007
cg24810735	*ANGPT1*	Body	Open sea	8	79.3±3.5	16.5±32.2	-62.8	1.95×10^{-5}	0.0003

Data are average beta-values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site.

Table 6. Pathways modulated by exercise training ($q \leq 0.05$).

Pathway name	Enrichment score	Enrichment *P*-value	Enrichment *q*-value	# of Genes	Pathway ID
Endocytosis	16.44	7.23×10^{-8}	1.22×10^{-5}	115	kegg pathway 211
HTLV-I infection	13.52	1.35×10^{-6}	0.0001	154	kegg pathway 251
Wnt signaling pathway	11.93	6.61×10^{-6}	0.0003	88	kegg pathway 142
Proteoglycans in cancer	11.71	8.21×10^{-6}	0.0003	122	kegg pathway 164
Pathways in cancer	11.15	1.44×10^{-5}	0.0005	171	kegg pathway 128
Axon guidance	10.8	2.04×10^{-5}	0.0006	77	kegg pathway 176
Gap junction	10.44	2.92×10^{-5}	0.0007	58	kegg pathway 127
Regulation of actin cytoskeleton	9.97	4.66×10^{-5}	0.001	120	kegg pathway 179
Glutamatergic synapse	9.05	0.0001	0.002	67	kegg pathway 182
Calcium signaling pathway	9.03	0.0001	0.001	101	kegg pathway 263
Hippo signaling pathway	9.02	0.0001	0.002	92	kegg pathway 20

MAPK signaling pathway	8.93	0.0001	0.002	129	kegg pathway 119
p53 signaling pathway	8.6	0.0002	0.002	32	kegg pathway 149
Melanogenesis	8.28	0.00025	0.003	64	kegg pathway 206
Cholinergic synapse	7.94	0.00035	0.004	69	kegg pathway 237
Amphetamine addiction	7.53	0.0005	0.006	37	kegg pathway 50
Focal adhesion	7.13	0.0008	0.008	103	kegg pathway 5
Circadian entrainment	7.07	0.0008	0.008	56	kegg pathway 51
Basal cell carcinoma	7.02	0.0009	0.008	39	kegg pathway 35
Dopaminergic synapse	6.99	0.0009	0.008	68	kegg pathway 91
Dilated cardiomyopathy	6.34	0.002	0.01	53	kegg pathway 134
Epstein-Barr virus infection	6.05	0.002	0.02	113	kegg pathway 252

Cocaine addiction	5.92	0.003	0.02	28	kegg pathway 107
Long-term potentiation	5.88	0.003	0.02	35	kegg pathway 208
Hedgehog signaling pathway	5.32	0.005	0.03	33	kegg pathway 167
Chronic myeloid leukemia	5.17	0.006	0.03	42	kegg pathway 240
Melanoma	5.17	0.006	0.03	42	kegg pathway 249
Fc gamma R-mediated phagocytosis	5.16	0.006	0.03	45	kegg pathway 77
Glioma	4.95	0.007	0.04	38	kegg pathway 161
B cell receptor signaling pathway	4.95	0.007	0.04	38	kegg pathway 188
ECM-receptor interaction	4.94	0.007	0.04	47	kegg pathway 41
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	4.92	0.007	0.04	17	kegg pathway 248
Other types of O-	4.92	0.007	0.04	17	kegg pathway 38

glycan biosynthesis					
PI3K-Akt signaling pathway	4.74	0.009	0.04	166	kegg pathway 45
Oocyte meiosis	4.69	0.009	0.04	60	kegg pathway 22
T cell receptor signaling pathway	4.6	0.01	0.046	54	kegg pathway 70
Aminoacyl-tRNA biosynthesis	4.59	0.01	0.046	27	kegg pathway 116
Spliceosome	4.5	0.01	0.049	75	kegg pathway 131
Small cell lung cancer	4.48	0.01	0.049	42	kegg pathway 114
Progesterone-mediated oocyte maturation	4.36	0.01	0.05	50	kegg pathway 254

Legend: #, number.

Table 7. *MIR21* and *MIR210* DNA methylation after four weeks of exercise training (q<0.05).

Location in relation to					***DNA methylation (%)***				
CpG	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before Exercise**	**After exercise**	**Diff**	***P*-value**	***q*-value (≤0.005)**
cg27023597	*MIR21*	TS1500	Open Sea	17	67.4±3.6	64.4±4.0	-3	0.007	0.01
cg04276626	*MIR21*	TSS200	Open Sea	17	79.3±3.4	76	-3.3	0.008	0.01
cg02515217	*MIR21*	TSS200	Open Sea	17	80.0±2.4	78.5±2.1	-1.5	0.009	0.01
cg07181702	*MIR21*	Body	Open	17	78.4±3.0	74.6±3.9	-3.8	0.02	0.03

			Sea						
cg02471760	*MIR210*	TSS1500	S Shore	11	54.6±4.7	50.4±4.6	-4.2	0.002	0.004
cg08200293	*MIR210*	TSS1500	Island	11	13.2±1.2	11.8±2.1	-1.4	0.01	0.02
cg05858042	*MIR210*	TSS1500	Island	11	24.5±3.9	21.7±5.1	-2.7	0.005	0.009
cg07410811	*MIR210*	TSS1500	Island	11	8.1±1.2	6.7±1.5	-1.4	0.003	0.007
cg15482500	*MIR210*	TSS200	Island	11	11.6±0.7	9.3±0.7	-2.3	9.39×10^{-5}	0.0007
cg01277369	*MIR210*	TSS200	Island	11	7.5±1.1	6.2±0.9	-1.3	0.007	0.01
cg03880841	*MIR210*	Body	Island	11	18.5±0.01	15.9±1.2	-2.6	6.2×10^{-5}	0.0005

Data are average beta-values ± standard deviation.

Legend: Chr, chromosome number; Diff, difference; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site.

Chapter 7 Genome-wide sperm DNA methylation changes after 3 months of exercise training in humans – published in *Epigenomics*, 2015

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GENOME-WIDE SPERM DNA METHYLATION CHANGES AFTER THREE MONTHS OF EXERCISE TRAINING IN HUMANS

Joshua Denham[1], Brendan J. O'Brien[2], Jack T. Harvey[2], Fadi J. Charchar[1*]

[1] Faculty of Science and Technology, Federation University Australia, Mt Helen, Victoria, Australia.

[2] Faculty of Health, Federation University Australia, Mt Helen, Victoria, Australia.

*Corresponding author: Prof Fadi Charchar, Y Building, University Drive, Federation University Australia, Mt Helen, Victoria, Australia 3350. Ph: +613 5327 6098, Fax: +613 5327 9602, Email: f.charchar@federation.edu.au

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Abstract

Aim: DNA methylation programs gene expression and is involved in numerous biological processes. Accumulating evidence supports transgenerational inheritance of DNA methylation changes in mammals via germ cells. Our aim was to determine the effect of exercise on sperm DNA methylation.

Materials and Methods: Twenty-four men were recruited and assigned to an exercise intervention or control group. Clinical parameters were measured and sperm samples were donated by subjects before and after the three month time-period. Mature sperm global and genome-wide DNA methylation was assessed using an ELISA assay and the 450K BeadChip (Illumina).

Results: Global and genome-wide sperm DNA methylation was altered after three months of exercise training. DNA methylation changes occurred in genes related to numerous diseases such as Schizophrenia and Parkinson's disease.

Conclusions: Our study provides the first evidence showing exercise training reprograms the sperm methylome. Whether these DNA methylation changes are inherited to future generations warrants attention.

Executive summary

Aim

- To determine the impact of exercise training on human sperm DNA methylation.

Materials and Methods

- Twenty-four healthy young men were recruited and allocated to an exercise training or control group.
- Health and fitness parameters were measured and sperm samples were collected before and after three months.
- Global and genome-wide DNA methylation was quantified using an ELISA assay and the Infinium HumanMethylation450 BeadChip (Illumina), respectively.
- Significant alterations in CpG methylation and corresponding gene pathways modulated by exercise training were analysed using Partek (Genomic Suite). The disease association was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).

Discussion

- Exercise training was associated with global demethylation of human sperm.
- DNA methylation changes occurred across the genome after exercise training in genes enriched for numerous developmental processes and pathways associated with the adaptation to exercise training.

- Genes related to disease were methylated after exercise, indicating transcriptional silencing.

Conclusions

- We provide the first evidence demonstrating exercise training regulates the human sperm methylome at CpG sites in genes associated with disease.

Introduction

DNA methylation is crucial for long-term gene expression regulation [41,585]. DNA methylation is, however, reprogrammable through environmental conditions [586,587]. Aberrant DNA methylation signatures are associated with age-related diseases such as cancer [588-590], atherosclerosis [53,591], type 2 diabetes mellitus [51,592], Alzheimer's disease [593] and major psychosis disorders [594,595].

Mounting evidence indicates that environmentally induced DNA methylation changes are inherited by subsequent generations and affects their phenotypes [58,59,596-601]. A likely mechanism for inheritance of DNA methylation profile is via the germ cells. Interestingly, aging is associated with sperm DNA methylation changes and may influence disease risk of future generations [602,603]. The differentially methylated regions in sperm obtained from old (12–14 month-old) versus young (3-month-old) mice were observed in the brain of the subsequent offspring, causing altered gene expression and increased behaviors associated with psychiatric disorders (schizophrenia and autism spectrum disorder) [603]. Although aging

has been investigated in context with sperm DNA methylation, the influence of other favorable environmental stimuli are yet to be determined.

We recently found that exercise training significantly improves cardiorespiratory fitness and lipid profile in conjunction with genome-wide leukocyte methylation changes in young men [604]. Others have shown that exercise training modulates skeletal myocyte [522,605,606] and adipocyte [523] DNA methylation as well as improving health and fitness phenotypes. Thus, somatic cells are vulnerable to epigenetic changes caused by exercise training, but whether exercise training regulates DNA methylation is germ cells (oocyte or sperm) is currently unknown.

The aim of this study was to determine the impact of exercise training on human sperm DNA methylation. Considering the mounting evidence supporting exercise training as an environmental modulator of somatic cell DNA methylation [522,523,604-606], we hypothesized that exercise training would significantly modify sperm DNA methylation in genes related to human diseases.

Materials and methods

Subjects

Twenty-four healthy young men were recruited for this study. Subjects were non-smoking, not taking any medications and free from any age-related chronic diseases, according to self-administered health questionnaires. Subjects were eligible if they had not engaged in any structured high-intensity aerobic exercise training during the past six months and were recruited predominantly from the University by flyers and by word-of-mouth.

The subjects' characteristics are outlined in Table 1. All subjects gave written informed consent to participate and this study was approved by the Federation University Australia Human Research Ethics Committee.

Procedures

On the initial day of testing, subjects were asked to complete health and physical activity questionnaires in order to establish their eligibility to participate in the study. Subjects were also familiarized with the testing procedures to follow and were given a collection tube for their sperm donation.

The second testing session involved a sperm donation, blood donation, physical measures and a test of maximal oxygen uptake ($\dot{V}O_{2max}$) on a treadmill. All testing sessions were performed in the morning (7–10 AM). Height, weight and body mass index were recorded using scales and a stadiometer. Blood pressure was measured and augmentation index was assessed by non-invasive applanation tonometry using the SphygmoCor instrument (AtCor, Australia). Subjects completed a maximal treadmill exercise test to assess $\dot{V}O_{2max}$. After a three minute warm-up, subjects began running at 10 $km \cdot h^{-1}$ and the treadmill speed was progressively increased by 1 $km \cdot h^{-1}$ every second minute until the volitional exhaustion or the supervising Exercise Physiologist deemed it appropriate to stop the test. Participants were fitted with a two-way breathing valve (Hans Rudolph) and breath-by-breath analysis was performed by the online metabolic system (Moxus, Modular, USA). $\dot{V}O_{2max}$ was determined as the highest O_2 value averaged over 60 s. Maximal treadmill speed was deemed the highest speed successfully completed and was adjusted for time so that participants were encouraged to exercise to their limit. The same testing procedures were completed 3–5 days after the cases' final exercise session or three months after their initial donation for controls.

Sperm and blood processing

Subjects gave resting preprandial sperm and blood donations before and after the three month exercise intervention. Following an overnight fast, subjects extracted their sperm by masturbating at home and donated their sample to the researchers at the University within 30 min of extraction. All sperm donations and analyses were performed according the World Health Organization's recommendations. Sperm cell number and vitality was assessed by the same researcher using a sheep-counter. Mature, motile sperm were isolated from lymphocytes, epithelial cells, immature or abnormal sperm and other cellular debris using the PureSperm 40/80 reagents (Nidacon). Briefly, 2 ml of PureSperm 40 was carefully layered on top of PureSperm 80, followed by gently aliquoting 1.5 ml of ejaculate onto the density gradient. The density gradient was spun at 300 G for 20 min. The mature sperm were pelleted and supernatant was removed. The pellet was washed twice by resuspending it in 5 ml of PBS before being spun at 500 G for 10 min. DNA was extracted from mature sperm suspended in 200 µl of PBS using the Purelink Genomic DNA Mini kit (LifeTechnologies) following the manufacturer's recommendations with some small adjustments. To deform the tightly packaged DNA in mature sperm, 0.03 mmol of DL-Dithiothreitol solution (Sigma-Aldrich) was added to the sample before the addition of proteinase K solution and the protein digestion step (incubation at 55°C) was extended from the recommended 10 min to 90 min. All extractions were performed immediately after sperm donation to prevent any temporal *de novo* influences on DNA methylation. DNA yield was assessed using the Nanodrop 2000 Spectrophotometer (Thermo Scientific) before being stored at -20°C.

A preprandial blood sample (24 ml) was drawn from the antecubital vein into serum separating tubes with the subject seated, using standard phlebotomy procedures. Blood was immediately stored on ice before further processing. Blood lipid assays were performed as described previously [604].

Global DNA methylation analysis

Average global DNA methylation of sperm from all subjects was quantified using the 5-mC ELISA Kit (Zymo Research, USA). All experiments were performed according to the manufacturer's procedures. All paired samples before and after the intervention were run on the same plate, as well as 100% methylated and 0% methylated controls, all run in duplicate with a standard curve. Absorbance was measured at 450 nm using the Multiskan FC Microplate Photometer (Thermo Scientific, Australia) and average DNA methylation was calculated using the formula generated from the standard curve. The average intra-assay coefficient of variation for duplicate samples was 4.85%.

Genome-wide DNA methylation analysis

Genome-wide sperm DNA methylation was quantified in 12 cases before and after the exercise intervention using the Infinium HumanMethylation 450K BeadChip (Illumina) as previously described [604]. Experiments were conducted following the manufacturer's guidelines, with the help of the Busselton Population Medical Research Institute. Briefly, raw *β*-values underwent Subset-quantile Within Array Normalization (SWAN) and were transformed to M-values using the logit function ($\log^2(\beta/(1-\beta))$) before analyzing differentially methylated CpG sites after exercise training. M-values were converted to *β*-values for representation of differentially methylated CpG sites in tables and graphs because they correspond to the percentage of CpG methylation and are a good biologically relevant representation. Genome-wide DNA methylation data was analyzed using Partek (Genomic Suite, version 6.6).

Exercise training

Subjects were randomly allocated to either an exercise training (cases) or control group. Cases completed sprint interval training twice weekly for three months. Three months of exercise training was chosen because it covered one human spermatogenesis cycle. The

controls did not engage in any form of training but were asked not to deviate from their routine physical activity habits. All subjects were asked to adhere to their normal diet. The sprint interval training schedule is outlined in Table 2. Sprint interval training is an effective type of exercise training that rapidly improves cardiorespiratory fitness and endurance performance [531,607-609].

The exercise training protocol of this study was based on research shown to improve cardiorespiratory fitness and endurance performance [604,610], but with some adjustments, to avoid overuse injuries that could occur with an extended training period (three months). Subjects completed 30 s sprints at maximal intensity with a passive four minute rest period between efforts. Training began with three sprints and was progressed by one effort every fortnight until week 7, when sprints were kept at six efforts for the remaining six weeks of the intervention. Before each session, subjects completed a short warm-up entailing a light 5 min jog, dynamic stretches and some 20 m sprints at 70, 80, 90 and 100% of maximal effort. Training was performed on the University's athletics oval and subjects were supervised and given constant encouragement during sprints by an Exercise Physiologist.

Statistical analyses

Kolmogorov-Smirnov and Shapiro-Wilks were performed to assess normality. Two-tailed paired *t*-tests were used to assess within-subject changes to physical, health and fitness variables. A repeated measures ANOVA was used to determine the interaction between global sperm DNA methylation before and after the intervention in relation to cases and controls using IBM SPSS for Windows (version 21). Differentially methylated CpG sites in cases were determined by repeated measures ANOVA using Partek (Genomic Suite, version 6.6). In order to control for false positives, a false discovery rate correction (FDR, $q \leq 0.1$) was applied to genome-wide data by converting *p*-values to *q*-values. Hierarchical clustering, gene ontology and pathway analysis on genome-wide CpG methylation data were performed

using Partek (Genomic Suite, version 6.6). Disease annotation analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7). Statistical significance was set at $p<0.05$.

Results

Exercise training improved cardiorespiratory fitness

To determine whether exercise training alters human sperm DNA methylation, 13 untrained male volunteers completed three months of exercise training using a modified training protocol (Table 2) previously shown to improve cardiorespiratory fitness and induce genome-wide DNA methylation changes in leukocytes [604]. Although $\dot{V}O_{2max}$ was unchanged (2.2%, $p=0.35$), relative to baseline, the cases, on average, had a significantly lower resting heart rate by 10 beats·min^{-1} and completed an extra one min at a significantly higher maximal treadmill speed ($p=0.01$ and $p=0.002$, respectively), while controls showed no significant changes in these variables (Table 1).

To assess whether exercise training influences average sperm DNA methylation, we assessed global sperm DNA methylation in cases and controls using ELISA-based chemistry. After adjusting for ELISA plate, relative to the unchanged global sperm DNA methylation observed in controls (n=11, 4.09%), sperm DNA was significantly de-methylated after three months of exercise training (n=13, -6.63%, interaction $p=0.006$) (Figure 1A and B).

Based on the global DNA methylation results we only quantified genome-wide sperm DNA methylation in 12 cases before and after three months of exercise training using the 450K BeadChip (Illumina). After applying a FDR ($q\leq0.1$), we found DNA methylation changes occurred throughout the genome in relation to the nearest gene and CpG islands (Figure 1C

and D). Of these, 7509 CpG sites relating to 4602 genes had significant DNA methylation changes ranging from 0.3 to 6.2% after exercise training (Figure 1E and 2). More CpG sites were demethylated rather than methylated after exercise training (4275 vs 3233, respectively). The top 25 methylated and demethylated CpG sites after exercise training are outlined in Table 3.

To gain insight into the biological impact of the CpG methylation changes following exercise, we performed gene ontology and pathway analysis. Genes with altered CpG methylation were those enriched for crucial biological processes including developmental process, anatomical structure, embryonic morphogenesis and organ development (Figure 3 and Supplementary Table 1). Pathway analysis revealed genes with CpG site DNA methylation changes were enriched for pathways such as MAPK signaling, PI3K-Akt signaling, pathways in cancer and ErbB signaling (Table 4).

In the search for the most biologically relevant CpG methylation changes, we focused on CpG sites with a ≥1.5% increase (n=703) or decrease (n=1247) in methylation after exercise training and entered them into DAVID for disease annotation analysis. While genes with demethylated CpG sites were those relating to normal variation, those with increased methylation were enriched for numerous diseases, with the most overrepresented diseases being Schizophrenia, Parkinson's Disease, autism, cervical cancer and leukemia (Figure 4).

The global erasure of sperm CpG methylation that occurs after fertilization is well established [611-613]. The sperm CpG methylation at imprinted genes, however, are resistant to the global erasure after fertilization [614,615] and as such, these genes are key candidates for possible transgenerational inheritance through sperm. We found 16 paternally imprinted genes were differentially methylated after the three month exercise training intervention (Table 5), in genes associated with pathologies including EKG abnormalities (*KCNQ1OT1* and *KCNQ1*),

autism and alcohol dependence (*GABRG3*), Tourette syndrome, obsessive compulsive disorder and dystonia (*SGCE*), and Alzheimer's disease, obesity, type 2 diabetes mellitus, blood pressure and atherosclerosis (*IGF2* and *INS-IGF2*).

Discussion

Unlike the genome, the epigenome is malleable to changing environments and these changes are somewhat heritable [586,587]. Germ cells (sperm and oocytes) are likely vectors that transfer environmentally-affected DNA methylation profiles to future generations. We are the first to show the exercise training-induced DNA methylation changes in human sperm. We found exercise training significantly manipulated genome-wide DNA methylation and most strikingly, increased methylation in disease-associated genes indicating transcriptional silencing. Moreover, methylation changes occurred in paternally imprinted genes that are exempt from the DNA methylation erasure after fertilization [614,615].

Mounting evidence has demonstrated the capacity of exercise training to influence the human DNA methylome in numerous tissues, including skeletal myocytes [522,605,606], adipocytes [523] and leukocytes [604]. Exercise training or increased physical activity tends to be associated with a reduced level of somatic cell global DNA methylation. Leukocyte DNA methylation was anti-correlated to levels of physical activity in 509 older adults (>70 y) [550] and we previously reported more demethylated leukocyte CpG sites in response to a four week exercise training intervention in young men [604]. Furthermore, global and gene-specific demethylation occurred after acute exercise in human skeletal myocytes [526]. In the present study, we found global sperm DNA methylation was reduced after three months of exercise training, suggesting that exercise may systemically demethylate both somatic and germ cells. Interestingly, older adults have more heavily methylated global sperm DNA compared to their younger

counterparts [602,616]. Also defective human sperm exhibit nearly two-fold higher levels of global DNA methylation [617]. To that end, the global demethylation observed in our subjects after exercise training could be favorable, possibly counteracting the increased DNA methylation associated with ageing. This, in turn, could positively influence fertility though future studies are necessary.

Interestingly, exercise training was associated with sperm methylation changes to CpG sites in genes related to pathways and biological processes similar to those observed in skeletal muscle [522] and leukocytes [604] from previous studies. For example, pathways regulated by exercise training-induced leukocyte DNA methylation alterations [604] that were also observed in sperm include MAPK signaling, PI3K-Akt signaling and pathways in cancer. Similarly, overlapping pathways between skeletal muscle [522] and sperm pathways modulated by exercise training include MAPK signaling, insulin signaling and ErbB signaling. Therefore, it would seem that some exercise-responsive genes (CpG sites) are regulated by DNA methylation in human somatic cells and also in sperm.

The genes with CpG methylation changes in the present study were enriched for numerous molecular and cellular processes including anatomical structure development and morphogenesis, single-organism development and embryonic morphogenesis. Genes with exercise-induced CpG methylation changes were also associated with many debilitating diseases such as Schizophrenia, Parkinson's disease, cervical cancer, leukemia and autism. The genes associated with diseases were methylated after exercise training, indicating that these genes may have been transcriptionally silenced. Age-associated sperm CpG methylation patterns were observed in genes implicated in Schizophrenia in humans [602] and mice [603]. Some of the adverse DNA methylation patterns found in sperm of aged mice was transmitted to the brain of their offspring, leading to transcriptional dysregulation of genes involved in schizophrenia and autism, facilitating the disease-related traits [603]. Furthermore,

offspring (F1 to F3) of gestating female rats treated with endocrine disruptors (bisphenol-A, bis[2-ethlhexyl]phthalate and dibutyl phthalate and vinclozolin) have altered sperm DNA methylation and an increased risk of testis disease, obesity and infertility [58,66,467]. These environmentally-induced DNA methylation aberrations led to transcriptional changes in common pathways across numerous somatic cells, including those regulated in the present study (MAPK signaling, insulin signaling and pathways in cancer) [618]. Whether the altered sperm DNA methylation observed in our subjects will be transmitted to their offspring and prevent the development of disease is speculative and would require large human studies involving the analysis of multiple generations.

Germ cells undergo global demethylation following fertilization [611,615]. Consequently, the likelihood of such CpG methylation changes surviving this demethylation event is uncertain. We did, however, reveal a number of paternally imprinted genes (e.g. *IGF2*, *INS-IGF2*, *SGCE* and *GABRG3*) had differentially methylated CpGs after exercise training. These genes are exempt from the global demethylation after fertilization [614,615] and as such, are key candidates for exercise-induced transgenerational inheritance of DNA methylation gene programming. Our disease analysis using DAVID revealed the differentially methylated paternally imprinted genes after exercise training were related to neurological disorders (autism, Alzheimer's disease, Tourette's syndrome and obsessive compulsive disorder) and cardio-metabolic diseases (obesity, type 2 diabetes mellitus, high blood pressure and atherosclerosis). Whilst no evidence exists, it is tempting to speculate that exercise training may remodel the sperm methylome and that this epigenetic modification may reduce the disease risk of offspring.

DNA methyltransferase enzymes (DNMT1, DNMT3A, DNMT3B and DNMT3L) as well as ten-eleven translocation (TET1, TET2 and TET3) proteins could be modulated by exercise training leading to altered DNA methylation. Our study was not designed to target the exact

physiological or molecular mechanism by which exercise training influences DNA methylation in sperm, rather it was designed to investigate whether exercise training influences sperm DNA methylation. Therefore, further research is required to identify the molecular mechanisms evoking the changes in the sperm methylome after exercise training.

Our study has several limitations. Firstly, our study had a modest sample size and there was a statistically different global sperm DNA methylation between cases and controls at baseline. While we acknowledge this is not desirable, our statistical analysis accounted for the observed difference. We do, however, recommend that our results should be verified in a larger population. The relative exercise workload was not standardized. We did, however, ask that all subjects perform each sprint maximally without pacing to ensure they are working at maximal intensity. Certain diets are associated with DNA methylation levels in somatic cells [533,619]. Despite asking our subjects not to deviate from their routine dietary habits, the lack of control for diet in our study should also be acknowledged. Finally, we did not control for genotype-DNA methylation interactions or quantify DNA methylation in context with other epigenetic modifications or gene expression. Nonetheless, our novel finding that exercise alters human sperm DNA methylation should encourage future investigations into the influence of exercise training on germ cell epigenetic modifications and gene regulation.

Conclusions

We report for the first time that exercise training significantly modifies the human sperm methylome. Whilst exercise training was associated with an increased global sperm demethylation overall, methylation in key genes associated with human disease was increased indicating transcriptional silencing of these genes.

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Competing interests

The authors have no competing interests to disclose.

Figure Legends

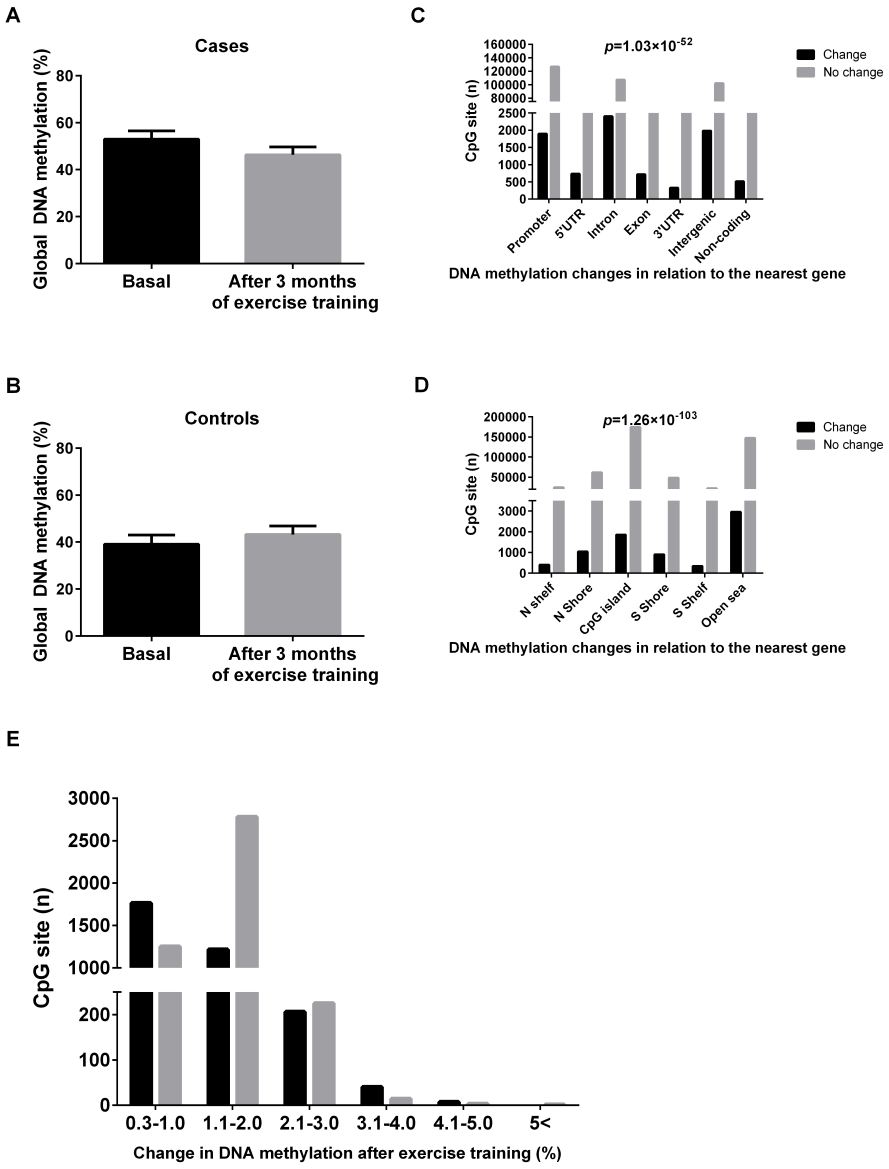

Figure 1. Sperm DNA methylation changes after exercise training. Average percent (±SE) of global sperm DNA methylation is given for 13 cases (A) and 11 controls (B) after the three month intervention. Relative to the controls who showed no global methylation change, cases exhibited a global demethylation (-6.63%) after three months of exercise training (mean±SE: 39.1±3.85 to 43.2±3.6 and 52.95±3.56 to 46.3±3.4, respectively, interaction p=0.006). Data are from repeated measures ANOVA adjusting for ELISA plate number. Genome-wide DNA methylation changes at CpG sites in relation to the nearest gene

(C) and CpG islands (D) are shown**.** Data are from Chi^2 tests. E) The number of CpG sites with increased methylation (black bars) and decreased methylation (grey bars) are given on the y-axis with the magnitude of change illustrated on the x-axis. Data are from differentially methylated CpG sites ($q \leq 0.1$).

Figures

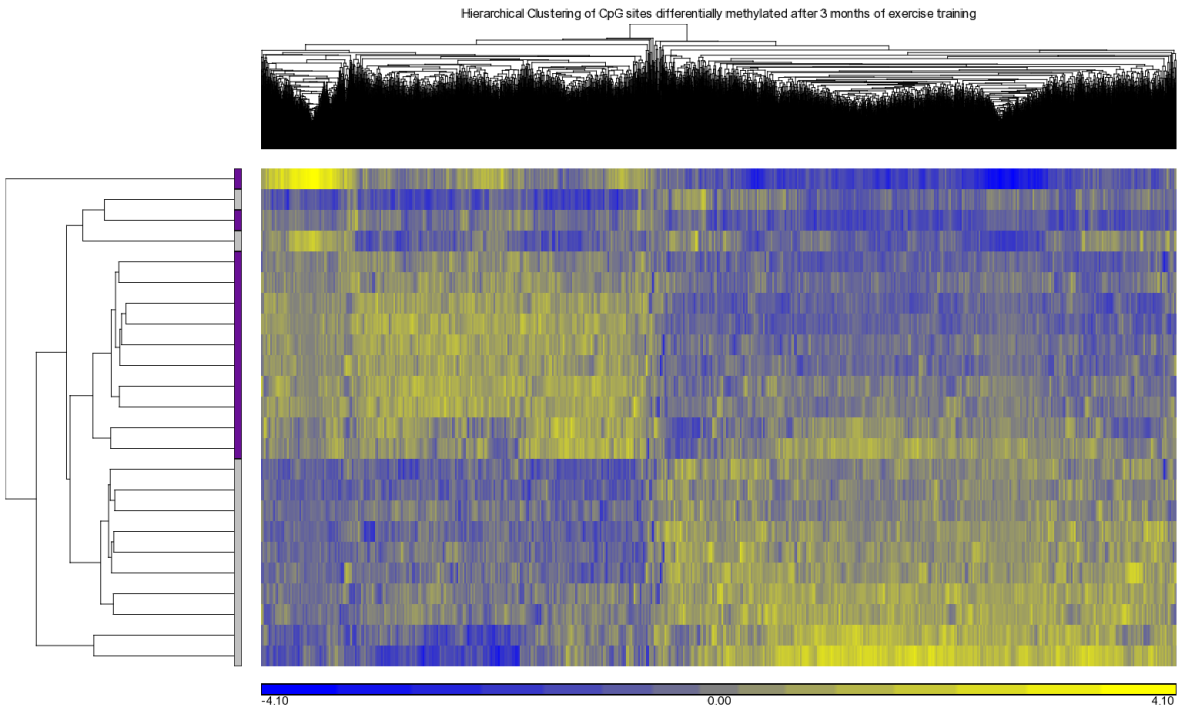

Figure 2. Hierarchical clustering of CpG sites after three months of exercise training. The CpG methylation status is indicated by blue (low methylation) and yellow (high methylation) from 12 subjects before (grey bar) and after (purple bar) three months of

exercise training. Each branch of the array tree on the left of the purple and grey bars are subjects. The array tree at the top of the hierarchical cluster indicates clusters of CpG sites modified by exercise training. Data are from differentially methylated CpG sites ($q \leq 0.1$).

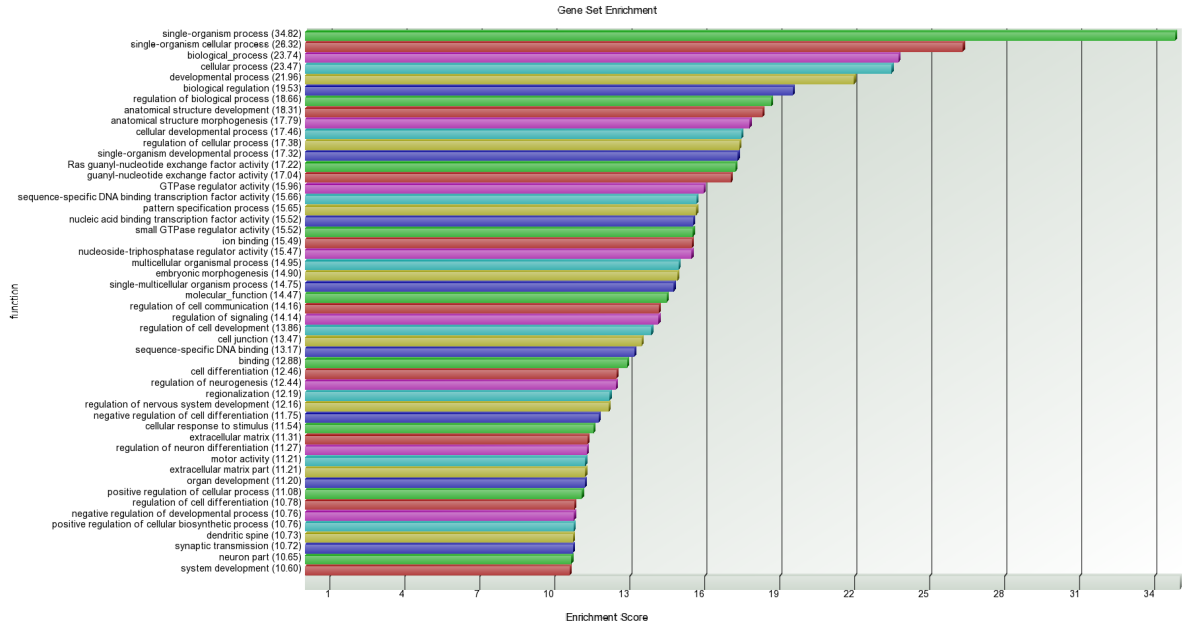

Figure 3. Gene ontology for genes with differentially methylated CpG sites after exercise training. Genes with altered CpG site methylation after exercise are grouped for function on the y-axis with corresponding enrichment scores on the x-axis. Data are from differentially methylated CpG sites ($q \leq 0.1$).

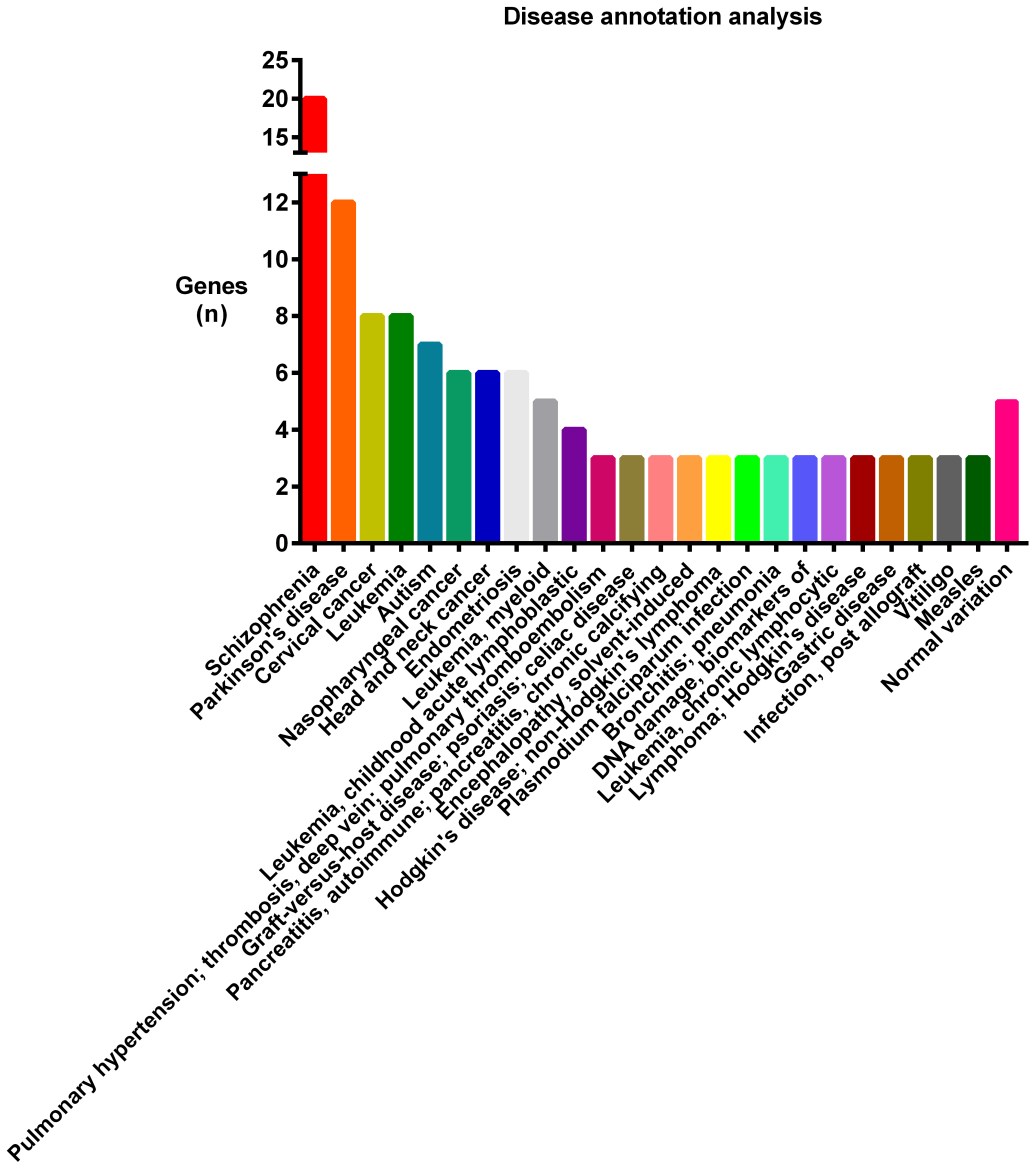

Figure 4. Disease annotation analysis for genes with differentially methylated CpG sites after exercise training. Data are from genes with a ≥1.5% increase or decrease in methylation after exercise training ($q \leq 0.1$). The y-axis represents the number of genes for each annotated disease (x-axis).

Table 1. Participant characteristics before and after three months of exercise training.

Variable	Cases (n=13)		Controls (n=11)	
	Before Exercise	After Exercise	Before	After
Age (y)	24.4±5.19		22.45±4.74	
Ht (cm)	181.1±6.54		177.95±4.07	
Wt (kg)	83.58±10.7	84.01±11.97	82.22±11.83	82.93±10.64
BMI (Wt/Ht^2)	25.53±3.34	25.89±3.61	25.94±3.5	25.9±3.08
HR ($beats \cdot min^{-1}$)	**78.54±15.64**	**68.23±7.15***	68.18±11.58	66.45±8.19
SBP (mm Hg)	130.46±11.82	129.85±10.64	123.45±9.2	123.09±10.24
DBP (mm Hg)	77.23±9.87	76.38±7.85	75.82±8.51	72.27±7.14
CSBP (mm Hg)	110.92±9.86	111.08±8.43	106.91±8.38	105.72±9.32
CDBP (mm Hg)	76.08±17.35	77.69±7.77	76.36±8.64	73.64±7.38
Aix (%)	-4.15±9.73	-4.15±8.73	-2.36±12.8	-1.18±11.71
Cholesterol (mmol/l)^	4.82±0.88	4.77±1.04	4.01±1.0	3.97±0.77
Triglyceride (mmol/l)^	1.01±0.48	1.07±0.46	0.9±0.38	0.82±0.18
HDLC (mmol/l)^	1.43±0.25	1.43±0.3	1.37±0.2	1.29±0.14
LDLC (mmol/l)^	2.92±0.79	2.87±0.88	2.22±0.75	2.32±0.74
Glucose (mmol/l)^	4.78±0.38	5.22±0.52	4.9±0.46	4.92±0.31
Insulin (mmol/l)^	6.94±2.72	10.72±6.44	7.15±3.65	12.78±16.42
$\dot{V}O_{2max}$ ($ml \cdot kg \cdot min^{-1}$)	47.43±5.64	48.47±6.0	45.85±7.34	44.92±5.49
Maximal treadmill speed ($km \cdot h^{-1}$)	**14.4±1.12**	**14.9±1.11****	14.07±1.52	13.84±1.8

Sperm volume (ml)	4.7±1.4	5.17±0.87	3.95±1.7	3.97±1.79
Sperm #	$1.35\times10^{9}\pm$ 1.21×10^{9}	$1.39\times10^{9}\pm$ 1.41×10^{9}	$1.58\times10^{9}\pm$ 1.56×10^{9}	$1.33\times10^{9}\pm$ 1.39×10^{9}
Sperm alive (n)	$9.12\times10^{8}\pm$ 1.09×10^{9}	$9.08\times10^{8}\pm$ 1.02×10^{9}	$8.36\times10^{8}\pm$ 1.07×10^{9}	$6.05\times10^{8}\pm$ 4.68×10^{8}
Sperm alive (%)	61.65±24.42	62.72±13.77	48.26±23.26	53.89±14.14

Legend: Ht, height; Wt, weight; BMI, body mass index; HR, resting heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; CSBP, central (aortic) blood pressure; CDBP, central (aortic) diastolic blood pressure; augmentation index; HDLC, high-density lipo-protein cholesterol; LDLC, low-density lipoprotein cholesterol; $\dot{V}O_{2max}$, maximal oxygen uptake; ^, n=8; #, number; %, percentage. Data are from two-tailed paired t-test and expressed as mean±SD. Sperm analysis was conducted on semen samples and not from highly motile sperm isolated using the PureSperm 40/80 reagents (Nidacon).

Table 2. Description of the three month exercise program.

Week	Session #	Training load*	Training sprint time (min)	Total session time (min)
1	1	3 sprints	1.5	9.5
	2	3 sprints	1.5	9.5
2	3	3 sprints	1.5	9.5
	4	3 sprints	1.5	9.5
3	5	4 sprints	2	14
	6	4 sprints	2	14
4	7	4 sprints	2	14
	8	4 sprints	2	14
5	9	5 sprints	2.5	18.5
	10	5 sprints	2.5	18.5
6	11	5 sprints	2.5	18.5
	12	5 sprints	2.5	18.5
7	13	6 sprints	3	23
	14	6 sprints	3	23
8	15	6 sprints	3	23

	16	6 sprints	3	23
9	17	6 sprints	3	23
	18	6 sprints	3	23
10	19	6 sprints	3	23
	20	6 sprints	3	23
11	21	6 sprints	3	23
	22	6 sprints	3	23
12	23	6 sprints	3	23
	24	6 sprints	3	23
	Total Time	120	60	454

Legend: # number; * all sprints were completed at maximum intensity, separated by a four minute passive recovery.

Table 3. DNA methylation changes after three months of exercise training ($q \leq 0.1$).

Location in relation to					***DNA methylation (%)***				
CpG site	**Closest gene**	**Gene region**	**CpG island**	**Chr**	**Before (mean±SD)**	**After exercise (mean±SD)**	**Δ**	***p*-value**	***q*-value (≤0.1)**
cg02598618	-	-	Open sea	10	94.0±1.4	98.6±1.1	4.6	1.79×10^{-5}	0.06
cg02655397	-	-	N shelf	11	80.7±4.4	85.1±3.3	4.5	0.0005	0.08
cg10243075	*CACNA1A*	Body	N shore	19	23.2±3.6	27.6±5.0	4.4	0.0004	0.08
cg27222157	*RBM14*	TSS1500	N shore	11	22.2±6.8	26.5±8.5	4.4	0.0009	0.08
cg10413297	*RFPL3S*	TSS1500	Open sea	22	25.6±6.7	29.7±6.6	4.1	0.001	0.09
cg16533336	*WBSCR17*	Body	Open sea	7	76.7±2.6	80.8±2.3	4.1	1.17×10^{-7}	0.02
cg09194930	*MT1L*	TSS200	Island	16	14.7±9.5	18.8±12.7	4.1	0.001	0.09
cg00685853	-	-	N shore	2	14.4±3.9	18.4±6.2	4.0	0.0001	0.07
cg27205941	*GMDS*	TSS200	Island	6	29.0±4.1	32.9±3.5	4.0	0.001	0.09

cg17745251	*C10orf140*	Body	Island	10	25.0±2.7	29.0±3.0	4.0	0.002	0.09
cg25485435	*RAMP1*	TSS1500	N shore	2	16.9±3.5	20.8±3.0	3.9	0.0002	0.07
cg11888420	-	-	Open sea	6	33.0±2.7	36.8±4.6	3.8	0.001	0.09
cg08715827	-	-	Open sea	12	6.1±2.3	9.8±5.8	3.8	0.001	0.09
cg15617548	*GORASP2*	5'UTR	Island	2	23.5±2.8	27.1±2.8	3.7	0.0009	0.08
		1stExon							
cg15488194	*MBNL1*	TSS1500	Open sea	3	23.9±11.9	27.5±12.8	3.7	0.002	0.097
		5'UTR							
cg17271592	*HIST2H2AA4*	TSS1500	Island	1	13.8±3.1	17.4±2.6	3.6	0.0006	0.08
	HIST2H2AA3								
cg06785129	*RFPL3*	TSS1500	Open sea	22	18.6±3.2	22.2±4.2	3.6	0.002	0.09
cg14047339	*RUNX2*	Body	Island	6	33.7±2.2	37.3±2.9	3.6	0.002	0.09
		5'UTR							
	SUPT3H	1stExon							
		5'UTR							
cg13916261	*FNBP1*	Body	N shelf	9	24.4±3.9	27.9±4.9	3.5	0.0003	0.08

cg09303484	-	-	Open sea	7	63.3±4.3	66.8±3.6	3.5	0.0008	0.08
cg05528975	-	-	Open sea	2	25.9±2.5	29.4±3.1	3.5	0.0005	0.08
cg17627829	*HCG18*	Body	N shore	6	9.5±3.4	13.0±4.1	3.5	0.002	0.09
cg05629323	*TFDP3*	3'UTR	Open sea	X	8.8±2.7	12.3±4.4	3.5	0.002	0.09
		1stExon							
cg20681184	*PIK3CD*	5'UTR	S shore	1	14.5±2.8	18.0±4.5	3.5	0.002	0.09
cg15069995	*HORMAD1*	5'UTR	Open sea	1	5.9±2.1	9.3±3.9	3.4	0.0009	0.08
cg13062455	*SEL1L*	Body	Open sea	14	88.2±2.3	85.4±3.3	-2.9	1.27×10^{-5}	0.05
cg24360197	-	-	Open sea	15	70.1±8.4	67.2±7.9	-2.9	0.001	0.09
cg03359503	*CLIC2*	5'UTR	Open sea	X	87.7±4.1	84.7±.5	-3.0	0.0009	0.08
		1^{st} Exon							
cg24121979	-	-	Open sea	12	94.9±3.2	91.9±2.0	-3.0	0.0003	0.08
cg20909579	*PDE6B*	TSS1500	Island	4	89.6±2.9	86.6±5.4	-3.0	0.0008	0.08
		Body							
cg02507663	*NCRNA00219*	Body	S shore	5	54.9±8.4	51.9±8.4	-3.0	0.001	0.09
	SNORA13	TSS200							

cg27569203	*CELSR1*	Body	Island	22	90.2±2.4	87.3±1.6	-3.0	0.002	0.09
cg26235273	*DKK3*	3'UTR	Open sea	11	71.0±4.0	67.8±3.8	-3.1	0.0006	0.08
cg17518550	*C1orf150*	TSS200	Open sea	1	82.3±4.0	79.2±6.4	-3.1	0.001	0.09
cg16514287	*SLC25A36*	Body	Open sea	3	94.0±1.4	90.8±1.1	-3.1	1.7×10^{-6}	0.03
cg26057840†	-	-	N shore	20	89.4±2.7	86.2±2.6	-3.2	0.0005	0.08
cg24544876	-	-	S shore	5	78.3±3.3	75.1±2.5	-3.2	0.0008	0.08
cg14630692	*URM1*	Body	Open sea	9	86.4±1.7	83.2±2.4	-3.2	0.001	0.09
cg03440485	-	-	Island	X	22.9±2.3	19.7±2.1	-3.2	0.001	0.09
cg15832577‡	*C1orf53*	Body	S shore	1	78.6±2.5	75.3±3.2	-3.3	0.0002	0.07
cg17494438	*DRGX*	TSS1500	N shelf	10	82.6±1.8	79.2±2.8	-3.3	0.001	0.09
cg16320838	*DNAJB7*	TSS1500	Open sea	22	92.5±14.5	89.0±19.1	-3.5	0.0005	0.08
	XPNPEP3	Body							
cg11342670	*KIAA0319*	Body	Open sea	6	78.9±9.3	75.5±10.3	-3.5	0.001	0.09
cg03987985	*L1TD1*	3'UTR	Open sea	1	75.6±4.6	72.0±5.6	-3.6	0.001	0.09
cg08377570	*LPAR6*	TSS1500	Open sea	13	87.5±2.1	83.8±2.5	-3.7	0.0005	0.07
	RB1	Body							

cg17486639	-	-	N shelf	19	89.8±1.6	86.0±3.1	-3.8	0.0006	0.08
cg03834767	*CDK14*	3'UTR	Open sea	7	90.6±2.0	86.4±6.0	-4.1	0.002	0.09
cg16631698	-	-	Island	10	81.7±4.2	77.2±2.3	-4.5	0.002	0.09
cg08269188	*CBX5*	5'UTR	Open sea	12	83.7±2.7	79.0±4.5	-4.7	5.17×10^{-5}	0.06
cg05946920	*GGA1*	Body	Island	22	39.7±6.7	33.6±3.4	-6.2	0.002	0.096

Data are expressed are beta values ± standard deviation.

Legend: Chr, chromosome number; Δ, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site; *DMR, differentially methylated region; †CDMR, cancer differentially methylated region; ‡RDMR, reprogrammed differentially methylated region.

Table 4. Molecular pathways related to genes with differentially methylated CpG sites after exercise training ($p \leq 0.05$).

Pathway Name	**Enrichment Score**	***p*-value**	**# genes**	**Pathway ID**
Circadian entrainment	8.0	0.0003	24	kegg pathway 51
Axon guidance	7.2	0.001	29	kegg pathway 176
Endocytosis	7.0	0.001	39	kegg pathway 211
Glutamatergic synapse	6.1	0.002	25	kegg pathway 182
Protein digestion and absorption	5.9	0.003	22	kegg pathway 46
ECM-receptor interaction	5.4	0.004	19	kegg pathway 41
GABAergic synapse	4.7	0.01	22	kegg pathway 133
Bile secretion	4.4	0.01	18	kegg pathway 74
MAPK signaling pathway	4.4	0.01	41	kegg pathway 119
Focal adhesion	4.3	0.01	34	kegg pathway 5
PI3K-Akt signaling pathway	4.3	0.01	54	kegg pathway 45
Huntington's disease	4.3	0.01	33	kegg pathway 53
Transcriptional misregulation in cancer	4.3	0.01	31	kegg pathway 94
African trypanosomiasis	4.1	0.02	10	kegg pathway 69

Colorectal cancer	4.1	0.02	13	kegg pathway 148
Viral myocarditis	3.9	0.02	15	kegg pathway 105
Taurine and hypotaurine metabolism	3.7	0.02	4	kegg pathway 195
Galactose metabolism	3.7	0.03	8	kegg pathway 15
Long-term depression	3.6	0.03	12	kegg pathway 250
Endocrine and other factor-regulated calcium reabsorption	3.6	0.03	10	kegg pathway 258
Retrograde endocannabinoid signaling	3.6	0.03	21	kegg pathway 129
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	3.4	0.03	7	kegg pathway 248
Nicotine addiction	3.4	0.03	10	kegg pathway 62
Allograft rejection	3.4	0.03	10	kegg pathway 177
Selenocompound metabolism	3.4	0.04	5	kegg pathway 135
Morphine addiction	3.2	0.04	20	kegg pathway 86
Amoebiasis	3.2	0.04	21	kegg pathway 56
Graft-versus-host disease	3.1	0.04	10	kegg pathway 209

Maturity onset diabetes of the young	3.1	0.04	7	kegg pathway 241
Pathways in cancer	3.1	0.05	49	kegg pathway 128
ErbB signaling pathway	3.1	0.05	15	kegg pathway 121
Type I diabetes mellitus	2.9	0.05	10	kegg pathway 242
Insulin secretion	2.9	0.05	17	kegg pathway 4

Table 5. Paternally imprinted gene CpG methylation changes caused by exercise training.

		Location in relation to			***DNA methylation (%)***				
Paternally imprinted gene	**CpG site**	**Gene region**	**CpG island**	**Chr**	**Basal (mean±SD)**	**After exercise (mean±SD)**	**Δ**	***p*-value**	***q*-value (≤0.1)**
GRB10	cg12358041	5'UTR Body	Open sea	7	92.3±1.2	91.0±1.2	-1.3	0.0004	0.08
	cg26050906	5'UTR TSS200	Open sea	7	91.8±1.1	90.9±1.1	-0.9	0.003	0.10
SGCE *PEG10*	cg22090863*	Body TSS1500	N shore	7	4.0±0.6	4.6±0.6	0.7	0.0007	0.08
SGCE *PEG10*	cg18139769	TSS1500 5'UTR	S shore	7	10.5±2.0	13.5±4.5	3.0	0.0006	0.08
DLGAP2	cg27316067	Body	S shore	8	64.3±11.2	62.2±11.3	-2.1	0.002	0.09

SFMBT2	cg15362304	Body	S shelf	10	93.4±1.1	92.5±1.1	-0.9	0.0007	0.08
INS-IGF2	cg00273464	Body	Open sea	11	89.8±1.7	87.7±1.2	-2.0	0.00002	0.06
IGF2		1stExon							
		5'UTR							
INS-IGF2	cg17665927	Body	Open sea	11	93.2±1.3	91.6±1.4	-1.5	0.0002	0.07
IGF2		1stExon							
		5'UTR							
INS-IGF2	cg05384664	Body	S shore	11	88.6±2.2	87.3±2.8	-1.4	0.001	0.09
IGF2		Body							
KCNQ1OT1	cg20406758‡	Body	Open sea	11	95.5±1.0	94.1±1.0	-1.4	0.001	0.09
KCNQ1		Body							
SLC38A4	cg12152384	Body	Open sea	12	93.4±1.0	92.3±0.9	-1.1	0.002	0.09
WIF1	cg15862358	TSS1500	S shore	12	6.7±2.6	8.6±5.5	1.9	0.001	0.09
DLK1	cg17412258	TSS1500	CpG island	14	2.0±0.5	2.8±0.6	0.8	0.0005	0.08
	cg06504820	TSS1500	N shore	14	21.5±6.7	24.1±7.6	2.5	0.0003	0.07
RTL1	cg23546343	1stExon	N shore	14	93.0±0.9	91.8±1.0	-1.2	0.002	0.10

MIR431		TSS200							
MIR433		TSS1500							
GABRG3	cg08325929	Body	S shelf	15	86.2±2.5	84.9±2.7	-1.3	0.003	0.10
ZIM2	cg16008476	Body	N shore	19	5.9±1.6	7.6±2.7	1.7	0.0006	0.08
HM13	cg06000530	Body	CpG island	20	4.0±1.0	5.6±2.2	1.6	0.002	0.10
PSIMCT-1		TSS200							

Data are expressed are beta values ± standard deviation.

Legend: Chr, chromosome number; Δ, difference; 3', three prime; 5', five prime; UTR, untranslated region; TSS1500, 1500 bases upstream of transcription start site; TSS200, 200 bases upstream of transcription start site; *DMR, differentially methylated region; ‡RDMR, reprogrammed differentially methylated region.

Supplementary Table 1. Gene ontology for genes with DNA methylation changes after exercise training ($q \leq 0.1$).

Function	**Type**	**Enrichment score**	***p*-value**	***q*-value**	**# genes**	**Go ID**
Single-organism process	BP	34.82	7.57×10^{-16}	3.88×10^{-12}	1428	44699
Single-organism cellular process	BP	26.32	3.70×10^{-12}	9.50×10^{-9}	1251	44763
Biological process	BP	23.74	4.89×10^{-11}	8.22×10^{-8}	1968	8150
Cellular process	BP	23.47	6.41×10^{-11}	8.22×10^{-8}	1678	9987
Developmental process	BP	21.96	2.89×10^{-10}	2.97×10^{-7}	591	32502
Biological regulation	BP	19.53	3.31×10^{-9}	2.83×10^{-6}	1227	65007
Regulation of biological process	BP	18.66	7.88×10^{-9}	5.78×10^{-6}	1168	50789
Anatomical structure development	BP	18.31	1.12×10^{-8}	7.18×10^{-6}	374	48856
Anatomical structure morphogenesis	BP	17.79	1.87×10^{-8}	1.07×10^{-5}	213	9653
Cellular developmental process	BP	17.46	2.62×10^{-8}	1.29×10^{-5}	312	48869
Regulation of cellular	BP	17.38	2.84×10^{-8}	1.29×10^{-5}	1113	50794

process						
Single-organism developmental process	BP	17.32	3.01×10^{-8}	1.29×10^{-5}	405	44767
Ras guanyl-nucleotide exchange factor activity	MF	17.22	3.32×10^{-8}	1.31×10^{-5}	36	5088
Guanyl-nucleotide exchange factor activity	MF	17.04	3.97×10^{-8}	1.45×10^{-5}	50	5085
Gtpase regulator activity	MF	15.96	1.18×10^{-7}	4.02×10^{-5}	96	30695
Sequence-specific DNA binding transcription factor activity	MF	15.66	1.58×10^{-7}	4.68×10^{-5}	182	3700
Pattern specification process	BP	15.65	1.60×10^{-7}	4.68×10^{-5}	84	7389
Nucleic acid binding transcription factor activity	MF	15.52	1.81×10^{-7}	4.68×10^{-5}	182	1071
Small gtpase regulator activity	MF	15.52	1.82×10^{-7}	4.68×10^{-5}	71	5083
Ion binding	MF	15.49	1.88×10^{-7}	4.68×10^{-5}	867	43167
Nucleoside-triphosphatase regulator	MF	15.47	1.91×10^{-7}	4.68×10^{-5}	97	60589

activity						
Multicellular organismal process	BP	14.95	3.22×10^{-7}	7.51×10^{-5}	435	32501
Embryonic morphogenesis	BP	14.90	3.37×10^{-7}	7.51×10^{-5}	81	48598
Single-multicellular organism process	BP	14.75	3.91×10^{-7}	8.37×10^{-5}	428	44707
Molecular function	MF	14.47	5.18×10^{-7}	0.0001	2015	3674
Regulation of cell communication	BP	14.16	7.09×10^{-7}	0.0001	319	10646
Regulation of signaling	BP	14.14	7.22×10^{-7}	0.0001	318	23051
Regulation of cell development	BP	13.86	9.59×10^{-7}	0.0002	124	60284
Cell junction	CC	13.47	1.42×10^{-6}	0.0003	136	30054
Sequence-specific DNA binding	MF	13.17	1.90×10^{-6}	0.0003	127	43565
Binding	MF	12.88	2.55×10^{-6}	0.0004	1597	5488
Cell differentiation	BP	12.46	3.88×10^{-6}	0.0006	218	30154
Regulation of neurogenesis	BP	12.44	3.96×10^{-6}	0.0006	80	50767

Regionalization	BP	12.19	5.08×10^{-6}	0.0008	53	3002
Regulation of nervous system development	BP	12.16	5.24×10^{-6}	0.0008	87	51960
Negative regulation of cell differentiation	BP	11.75	7.85×10^{-6}	0.001	88	45596
Cellular response to stimulus	BP	11.54	9.69×10^{-6}	0.001	653	51716
Extracellular matrix	CC	11.31	1.22×10^{-5}	0.002	65	31012
Regulation of neuron differentiation	BP	11.27	1.28×10^{-5}	0.002	67	45664
Motor activity	MF	11.21	1.35×10^{-5}	0.002	34	3774
Extracellular matrix part	CC	11.21	1.35×10^{-5}	0.002	44	44420
Organ development	BP	11.20	1.37×10^{-5}	0.002	159	48513
Positive regulation of cellular process	BP	11.08	1.55×10^{-5}	0.002	471	48522
Regulation of cell differentiation	BP	10.78	2.08×10^{-5}	0.002	174	45595
Negative regulation of developmental process	BP	10.76	2.12×10^{-5}	0.002	103	51093
Positive regulation of	BP	10.76	2.13×10^{-5}	0.002	210	31328

cellular biosynthetic process						
Dendritic spine	CC	10.73	2.19×10^{-5}	0.002	18	43197
Synaptic transmission	BP	10.72	2.22×10^{-5}	0.002	81	7268
Neuron part	CC	10.65	2.36×10^{-5}	0.002	105	97458
System development	BP	10.60	2.50×10^{-5}	0.003	115	48731
Response to external stimulus	BP	10.57	2.57×10^{-5}	0.003	168	9605
Synapse part	CC	10.35	3.21×10^{-5}	0.003	71	44456
Cell communication	BP	10.34	3.22×10^{-5}	0.003	148	7154
Signal transduction	BP	10.33	3.26×10^{-5}	0.003	538	7165
Axon guidance	BP	10.31	3.32×10^{-5}	0.003	68	7411
Negative regulation of neuron differentiation	BP	10.15	3.91×10^{-5}	0.003	18	45665
Neuron spine	CC	10.15	3.91×10^{-5}	0.003	18	44309
Cation binding	MF	10.14	3.94×10^{-5}	0.003	581	43169
Metal ion binding	MF	10.07	4.23×10^{-5}	0.004	572	46872
Positive regulation of biosynthetic process	BP	10.06	4.26×10^{-5}	0.004	211	9891

Positive regulation of macromolecule biosynthetic process	BP	10.05	4.33×10^{-5}	0.004	196	10557
Regulation of signal transduction	BP	10.04	4.38×10^{-5}	0.004	271	9966
Phospholipid binding	MF	9.97	4.66×10^{-5}	0.004	93	5543
Ion channel activity	MF	9.89	5.05×10^{-5}	0.004	73	5216
Anterior/posterior pattern specification	BP	9.83	5.40×10^{-5}	0.004	36	9952
Substrate-specific channel activity	MF	9.68	6.24×10^{-5}	0.005	74	22838
Rho guanyl-nucleotide exchange factor activity	MF	9.61	6.69×10^{-5}	0.005	22	5089
Negative regulation of signal transduction	BP	9.56	7.04×10^{-5}	0.005	110	9968
Cell part	CC	9.55	7.10×10^{-5}	0.005	1907	44464
Negative regulation of response to stimulus	BP	9.48	7.66×10^{-5}	0.006	131	48585
Positive regulation of nucleobase-containing compound metabolic	BP	9.45	7.84×10^{-5}	0.006	194	45935

process						
Regulation of axon extension involved in axon guidance	BP	9.42	8.13×10^{-5}	0.006	7	48841
Negative regulation of biological process	BP	9.26	9.50×10^{-5}	0.007	456	48519
Regulation of small gtpase mediated signal transduction	BP	9.26	9.55×10^{-5}	0.007	56	51056
Regulation of purine nucleotide metabolic process	BP	9.24	9.69×10^{-5}	0.007	72	1900542
Cell development	BP	9.12	0.0001	0.007	79	48468
Negative regulation of cell communication	BP	9.04	0.0001	0.008	114	10648
Gated channel activity	MF	9.03	0.0001	0.008	60	22836
Ion gated channel activity	MF	9.03	0.0001	0.008	60	22839
DNA binding	MF	9.00	0.0001	0.008	351	3677
Response to stimulus	BP	8.99	0.0001	0.008	827	50896

Positive regulation of nitrogen compound metabolic process	BP	8.94	0.0001	0.008	196	51173
Regulation of nucleotide metabolic process	BP	8.91	0.0001	0.008	72	6140
Channel activity	MF	8.88	0.0001	0.008	76	15267
Passive transmembrane transporter activity	MF	8.88	0.0001	0.008	76	22803
Organ morphogenesis	BP	8.80	0.0002	0.009	76	9887
Extracellular matrix disassembly	BP	8.78	0.0002	0.009	22	22617
Negative regulation of signaling	BP	8.74	0.0002	0.009	113	23057
Negative regulation of cellular process	BP	8.67	0.0002	0.01	421	48523
Neuron projection	CC	8.64	0.0002	0.01	80	43005
Cell projection part	CC	8.61	0.0002	0.01	111	44463
Positive regulation of cellular metabolic process	BP	8.61	0.0002	0.01	292	31325

Positive regulation of RNA metabolic process	BP	8.52	0.0002	0.01	173	51254
Regulation of cellular component organization	BP	8.51	0.0002	0.01	202	51128
Intracellular part	CC	8.51	0.0002	0.01	1607	44424
Enzyme regulator activity	MF	8.51	0.0002	0.01	161	30234
Positive regulation of metabolic process	BP	8.48	0.0002	0.01	306	9893
Regulation of Ras protein signal transduction	BP	8.45	0.0002	0.01	35	46578
Regulation of multicellular organismal process	BP	8.45	0.0002	0.01	275	51239
Cation channel activity	MF	8.40	0.0002	0.01	54	5261
Negative regulation of canonical Wnt receptor signaling pathway	BP	8.39	0.0002	0.01	22	90090
Collagen metabolic process	BP	8.39	0.0002	0.01	22	32963

Plasma membrane part	CC	8.38	0.0002	0.01	284	44459
Collagen	CC	8.37	0.0002	0.01	24	5581
Locomotion	BP	8.34	0.0002	0.01	159	40011
Regulation of multicellular organismal development	BP	8.27	0.0003	0.01	178	2000026
Regulation of Wnt receptor signaling pathway	BP	8.25	0.0003	0.01	40	30111
Collagen catabolic process	BP	8.25	0.0003	0.01	20	30574
Multicellular organismal metabolic process	BP	8.20	0.0003	0.01	24	44236
Regulation of localization	BP	8.14	0.0003	0.01	214	32879
Connective tissue development	BP	8.08	0.0003	0.01	26	61448
Positive regulation of transcription, DNA-dependent	BP	8.07	0.0003	0.01	164	45893
Positive regulation of	BP	8.06	0.0003	0.01	34	43547

gtpase activity						
Positive regulation of macromolecule metabolic process	BP	7.97	0.0003	0.02	284	10604
Neuron fate commitment	BP	7.96	0.0004	0.02	11	48663
Ionotropic glutamate receptor complex	CC	7.96	0.0004	0.02	11	8328
Synaptic membrane	CC	7.95	0.0004	0.02	45	97060
Multicellular organismal catabolic process	BP	7.93	0.0004	0.02	21	44243
Ion transmembrane transporter activity	MF	7.90	0.0004	0.02	127	15075
Cellular component organization	BP	7.86	0.0004	0.02	491	16043
Establishment of localization	BP	7.84	0.0004	0.02	427	51234
Embryonic placenta development	BP	7.84	0.0004	0.02	10	1892
Localization	BP	7.84	0.0004	0.02	71	51179
Tissue development	BP	7.83	0.0004	0.02	96	9888

Regulation of canonical Wnt receptor signaling pathway	BP	7.68	0.0005	0.02	30	60828
Energy reserve metabolic process	BP	7.67	0.0005	0.02	32	6112
Regulation of cellular component biogenesis	BP	7.67	0.0005	0.02	68	44087
Membrane-bounded vesicle	CC	7.66	0.0005	0.02	104	31988
Intracellular signal transduction	BP	7.65	0.0005	0.02	206	35556
Regulation of Rho protein signal transduction	BP	7.59	0.0005	0.02	23	35023
Axis specification	BP	7.52	0.0005	0.02	18	9798
Ion channel complex	CC	7.50	0.0006	0.02	41	34702
Microtubule motor activity	MF	7.48	0.0006	0.02	20	3777
Signaling	BP	7.46	0.0006	0.02	118	23052
Single organism signaling	BP	7.46	0.0006	0.02	118	44700

Negative regulation of Wnt receptor signaling pathway	BP	7.44	0.0006	0.02	27	30178
Embryonic organ morphogenesis	BP	7.44	0.0006	0.02	27	48562
Cell-cell signaling	BP	7.44	0.0006	0.02	115	7267
Positive regulation of gene expression	BP	7.39	0.0006	0.02	174	10628
Multicellular organismal macromolecule metabolic process	BP	7.32	0.0007	0.02	22	44259
Establishment or maintenance of cell polarity	BP	7.32	0.0007	0.02	22	7163
Regulation of developmental growth	BP	7.32	0.0007	0.02	26	48638
Lipid binding	MF	7.30	0.0007	0.02	123	8289
Regulation of cell proliferation	BP	7.29	0.0007	0.02	183	42127
Substrate-specific transmembrane	MF	7.22	0.0007	0.03	134	22891

transporter activity						
Regulation of developmental process	BP	7.21	0.0007	0.03	223	50793
Taxis	BP	7.16	0.0008	0.03	92	42330
Chemotaxis	BP	7.16	0.0008	0.03	92	6935
Ligand-gated ion channel activity	MF	7.15	0.0008	0.03	30	15276
Ligand-gated channel activity	MF	7.15	0.0008	0.03	30	22834
Cell fate determination	BP	7.15	0.0008	0.03	13	1709
Vesicle	CC	7.13	0.0008	0.03	116	31982
Negative regulation of cell development	BP	7.02	0.0009	0.03	27	10721
Wnt receptor signaling pathway	BP	7.02	0.0009	0.03	44	16055
Cellular component organization or biogenesis	BP	7.02	0.0009	0.03	491	71840
Positive regulation of biological process	BP	7.00	0.0009	0.03	513	48518

Membrane	CC	6.94	0.0010	0.03	801	16020
Regulation of metabolic process	BP	6.92	0.0010	0.03	672	19222
Positive regulation of transcription from RNA polymerase II promoter	BP	6.90	0.0010	0.03	118	45944
Negative regulation of axonogenesis	BP	6.90	0.0010	0.03	13	50771
Regulation of respiratory gaseous exchange	BP	6.89	0.0010	0.03	8	43576
Transport	BP	6.87	0.0010	0.03	416	6810
Transport vesicle membrane	CC	6.86	0.0011	0.03	17	30658
Energy derivation by oxidation of organic compounds	BP	6.85	0.0011	0.03	37	15980
Positive regulation of cell development	BP	6.84	0.0011	0.03	33	10720
Regulation of cell adhesion	BP	6.82	0.0011	0.03	51	30155
Actin filament-based	BP	6.82	0.0011	0.03	54	30029

process						
Cell surface receptor signaling pathway	BP	6.81	0.0011	0.03	312	7166
Cell-cell junction	CC	6.79	0.0011	0.03	52	5911
Activation of Ras gtpase activity	BP	6.79	0.0011	0.03	10	32856
Chromatin binding	MF	6.73	0.0012	0.04	62	3682
Tissue morphogenesis	BP	6.72	0.0012	0.04	63	48729
Regulation of nucleobase-containing compound metabolic process	BP	6.70	0.0012	0.04	487	19219
Negative regulation of neurogenesis	BP	6.69	0.0012	0.04	22	50768
Microtubule associated complex	CC	6.64	0.0013	0.04	29	5875
Transcription, DNA-dependent	BP	6.59	0.0014	0.04	308	6351
Early endosome membrane	CC	6.57	0.0014	0.04	21	31901

Cation channel complex	CC	6.52	0.0015	0.04	29	34703
Positive regulation of neurogenesis	BP	6.49	0.0015	0.04	26	50769
Coated vesicle membrane	CC	6.49	0.0015	0.04	25	30662
Columnar/cuboidal epithelial cell development	BP	6.42	0.0016	0.05	7	2066
Regulation of steroid biosynthetic process	BP	6.42	0.0016	0.05	13	50810
ER to Golgi transport vesicle membrane	CC	6.42	0.0016	0.05	11	12507
Dynein complex	CC	6.40	0.0017	0.05	12	30286
Positive regulation of cellular component organization	BP	6.40	0.0017	0.05	88	51130
Site of polarized growth	CC	6.39	0.0017	0.05	22	30427
Skeletal system morphogenesis	BP	6.37	0.0017	0.05	23	48705
Negative regulation of intracellular protein	BP	6.37	0.0017	0.05	23	10741

kinase cascade						
Common myeloid progenitor cell proliferation	BP	6.33	0.0018	0.05	3	35726
Regulation of somitogenesis	BP	6.33	0.0018	0.05	3	14807
Detection of monosaccharide stimulus	BP	6.33	0.0018	0.05	3	34287
Detection of glucose	BP	6.33	0.0018	0.05	3	51594
Detection of carbohydrate stimulus	BP	6.33	0.0018	0.05	3	9730
Detection of hexose stimulus	BP	6.33	0.0018	0.05	3	9732
Insulin-like growth factor-activated receptor activity	MF	6.33	0.0018	0.05	3	5010
Laminin-10 complex	CC	6.33	0.0018	0.05	3	43259
Cell projection	CC	6.26	0.0019	0.05	139	42995
Multicellular organismal development	BP	6.25	0.0019	0.05	81	7275

Positive regulation of Ras gtpase activity	BP	6.23	0.0020	0.05	23	32320
Endoplasmic reticulum part	CC	6.22	0.0020	0.05	142	44432
Regulation of GTP catabolic process	BP	6.21	0.0020	0.05	44	33124
Regulation of nitrogen compound metabolic process	BP	6.20	0.0020	0.05	495	51171
Neuron recognition	BP	6.19	0.0020	0.05	10	8038
Negative regulation of cell proliferation	BP	6.17	0.0021	0.05	88	8285
Regulation of cellular metabolic process	BP	6.16	0.0021	0.05	612	31323
Negative regulation of axon extension involved in axon guidance	BP	6.14	0.0022	0.05	5	48843
Negative regulation of JAK-STAT cascade	BP	6.14	0.0022	0.05	5	46426
N-methyl-D-aspartate selective glutamate	CC	6.14	0.0022	0.05	5	17146

receptor complex						
Regulation of axonogenesis	BP	6.10	0.0022	0.05	22	50770
Neuron differentiation	BP	6.09	0.0023	0.06	38	30182
Cytoskeleton organization	BP	6.05	0.0024	0.06	93	7010
Regulation of growth	BP	6.05	0.0024	0.06	85	40008
Regulation of nucleoside metabolic process	BP	6.01	0.0025	0.06	48	9118
Establishment of protein localization to membrane	BP	5.98	0.0025	0.06	9	90150
Integral to endoplasmic reticulum membrane	CC	5.97	0.0025	0.06	21	30176
Growth cone	CC	5.97	0.0025	0.06	21	30426
Cartilage development involved in endochondral bone morphogenesis	BP	5.97	0.0025	0.06	6	60351
Enzyme linked receptor protein signaling	BP	5.96	0.0026	0.06	112	7167

pathway						
Intramembranous ossification	BP	5.93	0.0027	0.06	4	1957
Direct ossification	BP	5.93	0.0027	0.06	4	36072
Regulation of transport	BP	5.91	0.0027	0.06	157	51049
Embryonic skeletal system morphogenesis	BP	5.90	0.0027	0.06	18	48704
Regulation of catabolic process	BP	5.90	0.0027	0.06	88	9894
Regulation of gtpase activity	BP	5.90	0.0027	0.06	43	43087
Transmembrane receptor protein tyrosine kinase signaling pathway	BP	5.90	0.0028	0.06	87	7169
Extracellular vesicular exosome	CC	5.85	0.0029	0.07	16	70062
Antigen processing and presentation of exogenous peptide antigen via MHC class II	BP	5.83	0.0029	0.07	21	19886
Tight junction	CC	5.83	0.0029	0.07	23	5923

Occluding junction	CC	5.83	0.0029	0.07	23	70160
Serine-type endopeptidase inhibitor activity	MF	5.83	0.0029	0.07	22	4867
Cellular component movement	BP	5.83	0.0029	0.07	138	6928
Membrane-enclosed lumen	CC	5.78	0.0031	0.07	101	31974
Vesicle membrane	CC	5.77	0.0031	0.07	61	12506
Regulatory region nucleic acid binding	MF	5.77	0.0031	0.07	62	1067
Regulatory region DNA binding	MF	5.77	0.0031	0.07	62	975
Regulation of primary metabolic process	BP	5.76	0.0031	0.07	601	80090
Regulation of purine nucleotide catabolic process	BP	5.72	0.0033	0.07	47	33121
Cartilage development	BP	5.72	0.0033	0.07	19	51216
Response to glucagon stimulus	BP	5.71	0.0033	0.07	12	33762

Regulation of anatomical structure morphogenesis	BP	5.70	0.0034	0.07	98	22603
Cytoplasmic vesicle membrane	CC	5.65	0.0035	0.07	59	30659
Regulation of biological quality	BP	5.65	0.0035	0.07	314	65008
Regulation of nucleotide catabolic process	BP	5.64	0.0035	0.07	47	30811
Metal ion transmembrane transporter activity	MF	5.64	0.0036	0.08	65	46873
Ionotropic glutamate receptor activity	MF	5.62	0.0036	0.08	7	4970
Cell motility	BP	5.60	0.0037	0.08	102	48870
Regulation of stress-activated MAPK cascade	BP	5.60	0.0037	0.08	31	32872
Single-organism transport	BP	5.59	0.0037	0.08	323	44765
Regulation of cellular	BP	5.58	0.0038	0.08	111	60341

localization						
Antigen processing and presentation of peptide antigen via MHC class II	BP	5.56	0.0038	0.08	21	2495
Response to endogenous stimulus	BP	5.56	0.0039	0.08	156	9719
Actin cytoskeleton organization	BP	5.55	0.0039	0.08	44	30036
Anchoring collagen	CC	5.55	0.0039	0.08	5	30934
Axonal growth cone	CC	5.55	0.0039	0.08	5	44295
Mannose metabolic process	BP	5.55	0.0039	0.08	5	6013
Semaphorin-plexin signaling pathway	BP	5.52	0.0040	0.08	6	71526
Biological adhesion	BP	5.52	0.0040	0.08	121	22610
Cell adhesion	BP	5.52	0.0040	0.08	121	7155
Extracellular organelle	CC	5.51	0.0040	0.08	16	43230
Extracellular membrane-bounded organelle	CC	5.51	0.0040	0.08	16	65010
Regulation of stress-	BP	5.50	0.0041	0.08	31	70302

activated protein kinase signaling cascade						
Positive regulation of insulin secretion	BP	5.50	0.0041	0.08	12	32024
Macromolecule localization	BP	5.47	0.0042	0.08	57	33036
Intrinsic to endoplasmic reticulum membrane	CC	5.46	0.0043	0.08	23	31227
Homeostatic process	BP	5.45	0.0043	0.08	152	42592
Transmembrane transporter activity	MF	5.44	0.0043	0.08	141	22857
Endocytosis	BP	5.44	0.0044	0.08	46	6897
Negative regulation of cell adhesion	BP	5.43	0.0044	0.08	20	7162
Integral to lumenal side of endoplasmic reticulum membrane	CC	5.41	0.0045	0.09	9	71556
Embryonic skeletal system development	BP	5.41	0.0045	0.09	10	48706
Protein binding	MF	5.40	0.0045	0.09	965	5515

Small gtpase mediated signal transduction	BP	5.36	0.0047	0.09	71	7264
Regulation of steroid metabolic process	BP	5.35	0.0047	0.09	16	19218
Secretion by cell	BP	5.33	0.0048	0.09	63	32940
Liver development	BP	5.32	0.0049	0.09	17	1889
Antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	BP	5.30	0.0050	0.09	21	2504
Epithelial tube morphogenesis	BP	5.29	0.0050	0.09	19	60562
Regulation of response to stimulus	BP	5.28	0.0051	0.09	348	48583
Gtpase activator activity	MF	5.27	0.0051	0.09	47	5096
Organelle	CC	5.27	0.0051	0.09	1126	43226
Extracellular-glutamate-gated ion channel activity	MF	5.27	0.0051	0.09	7	5234
Osteoblast development	BP	5.27	0.0051	0.09	7	2076

Adherens junction	CC	5.25	0.0053	0.10	37	5912
Cardiac septum morphogenesis	BP	5.22	0.0054	0.10	11	60411
Cation transmembrane transporter activity	MF	5.22	0.0054	0.10	94	8324
Secretion	BP	5.22	0.0054	0.10	75	46903
Regulation of insulin secretion	BP	5.21	0.0054	0.10	29	50796
Regulation of peptide hormone secretion	BP	5.21	0.0055	0.10	31	90276
Regulation of hormone secretion	BP	5.19	0.0056	0.10	36	46883
Dorsal spinal cord development	BP	5.19	0.0056	0.10	4	21516
Peripheral nervous system myelin maintenance	BP	5.19	0.0056	0.10	4	32287
Positive regulation of superoxide anion generation	BP	5.19	0.0056	0.10	4	32930
Lipid particle	BP	5.19	0.0056	0.10	4	34389

organization						
Morphogenesis of an epithelium	BP	5.17	0.0057	0.10	50	2009
Positive regulation of hormone secretion	BP	5.17	0.0057	0.10	17	46887
Establishment of protein localization to plasma membrane	BP	5.17	0.0057	0.10	8	90002
Rab guanyl-nucleotide exchange factor activity	MF	5.17	0.0057	0.10	8	17112
Negative regulation of osteoblast differentiation	BP	5.17	0.0057	0.10	10	45668
Response to chemical stimulus	BP	5.16	0.0057	0.10	366	42221
Ras gtpase activator activity	MF	5.16	0.0057	0.10	25	5099
Response to nitrogen compound	BP	5.15	0.0058	0.10	96	1901698
Axonogenesis	BP	5.13	0.0059	0.10	24	7409
Regulation of JNK cascade	BP	5.13	0.0059	0.10	27	46328

Substrate-specific transporter activity	MF	5.12	0.0059	0.10	146	22892
Positive regulation of cell differentiation	BP	5.12	0.0060	0.10	81	45597
Cytoplasmic membrane-bounded vesicle	CC	5.11	0.0060	0.10	89	16023
RNA biosynthetic process	BP	5.11	0.0061	0.10	323	32774
Regulation of secretion	BP	5.10	0.0061	0.10	74	51046
Regulation of cell-substrate adhesion	BP	5.10	0.0061	0.10	23	10810
Neuropeptide receptor activity	MF	5.10	0.0061	0.10	12	8188
Establishment of cell polarity	BP	5.10	0.0061	0.10	12	30010
Cellular component morphogenesis	BP	5.09	0.0061	0.10	63	32989
Cell fate commitment	BP	5.09	0.0062	0.10	26	45165
Positive regulation of secretion	BP	5.09	0.0062	0.10	40	51047

Golgi apparatus part	CC	5.07	0.0063	0.10	103	44431
Response to organic nitrogen	BP	5.05	0.0064	0.10	92	10243
Regulation of sequestering of triglyceride	BP	5.05	0.0064	0.10	5	10889
Platelet-derived growth factor binding	MF	5.05	0.0064	0.10	5	48407
MHC class II receptor activity	MF	5.05	0.0064	0.10	5	32395
Positive regulation of neuron differentiation	BP	5.04	0.0065	0.10	16	45666
Small conductance calcium-activated potassium channel activity	MF	5.04	0.0065	0.10	3	16286
Cardiac endothelial cell differentiation	BP	5.04	0.0065	0.10	3	3348
Endocardial cell differentiation	BP	5.04	0.0065	0.10	3	60956
N-acetylgalactosaminyl-	MF	5.04	0.0065	0.10	3	50510

proteoglycan 3-beta-glucuronosyltransferase activity						
Positive regulation of transcription of Notch receptor target	BP	5.04	0.0065	0.10	3	7221
Glossopharyngeal nerve morphogenesis	BP	5.04	0.0065	0.10	3	21615
Extracellular matrix-cell signaling	BP	5.04	0.0065	0.10	3	35426
Immunoglobulin production involved in immunoglobulin mediated immune response	BP	5.04	0.0065	0.10	3	2381
Nerve growth factor processing	BP	5.04	0.0065	0.10	3	32455
Enzyme binding	MF	5.04	0.0065	0.10	162	19899
Cell recognition	BP	5.03	0.0066	0.10	17	8037
Regulation of peptide secretion	BP	5.02	0.0066	0.10	31	2791

Regulation of peptide transport	BP	5.02	0.0066	0.10	31	90087
Cytoplasmic vesicle part	CC	4.99	0.0068	0.10	73	44433
Skeletal system development	BP	4.97	0.0069	0.11	30	1501
Postsynaptic membrane	CC	4.97	0.0070	0.11	35	45211
Postsynaptic density	CC	4.96	0.0070	0.11	22	14069
Bone morphogenesis	BP	4.95	0.0071	0.11	7	60349
Synaptic transmission, glutamatergic	BP	4.95	0.0071	0.11	7	35249
Cortical actin cytoskeleton	CC	4.95	0.0071	0.11	7	30864
Cellular response to glucagon stimulus	BP	4.95	0.0071	0.11	10	71377
Negative regulation of growth	BP	4.94	0.0071	0.11	36	45926
Regulation of transcription from RNA polymerase II promoter	BP	4.92	0.0073	0.11	185	6357
Embryonic digit	BP	4.91	0.0074	0.11	12	42733

morphogenesis						
Regulation of extent of cell growth	BP	4.91	0.0074	0.11	12	61387
Potassium channel complex	CC	4.90	0.0075	0.11	16	34705
Voltage-gated potassium channel complex	CC	4.90	0.0075	0.11	16	8076
Regulation of JAK-STAT cascade	BP	4.90	0.0075	0.11	16	46425
Regulation of phosphorus metabolic process	BP	4.88	0.0076	0.11	186	51174
Gland development	BP	4.84	0.0079	0.12	22	48732
Anion binding	MF	4.84	0.0079	0.12	380	43168
Regulation of membrane potential	BP	4.83	0.0080	0.12	34	42391
Regulation of phosphate metabolic process	BP	4.81	0.0081	0.12	184	19220
Epithelial cell development	BP	4.80	0.0082	0.12	24	2064

Embryonic heart tube morphogenesis	BP	4.79	0.0083	0.12	14	3143
Inner ear morphogenesis	BP	4.79	0.0083	0.12	14	42472
Regulation of vesicle-mediated transport	BP	4.79	0.0084	0.12	40	60627
Clathrin-coated vesicle membrane	CC	4.77	0.0085	0.12	19	30665
Cell migration	BP	4.77	0.0085	0.12	91	16477
Central nervous system neuron differentiation	BP	4.77	0.0085	0.12	15	21953
Negative regulation of axon extension	BP	4.75	0.0086	0.12	6	30517
Protein kinase C activity	MF	4.75	0.0086	0.12	6	4697
Regulation of cellular catabolic process	BP	4.75	0.0086	0.12	75	31329
Response to growth factor stimulus	BP	4.75	0.0086	0.12	83	70848
Organic substance biosynthetic process	BP	4.75	0.0087	0.12	500	1901576
L-amino acid transmembrane	MF	4.73	0.0088	0.12	10	15179

transporter activity						
Embryo implantation	BP	4.73	0.0088	0.12	10	7566
Endocytic vesicle membrane	CC	4.73	0.0088	0.12	22	30666
Glycogen metabolic process	BP	4.73	0.0088	0.12	12	5977
Transcription-coupled nucleotide-excision repair	BP	4.73	0.0088	0.12	12	6283
Neuron projection morphogenesis	BP	4.72	0.0089	0.12	32	48812
Epidermis development	BP	4.69	0.0092	0.13	28	8544
Regulation of actin filament-based process	BP	4.68	0.0093	0.13	37	32970
Cellular response to growth factor stimulus	BP	4.68	0.0093	0.13	81	71363
Response to nicotine	BP	4.67	0.0094	0.13	9	35094
Midbrain development	BP	4.67	0.0094	0.13	9	30901
Cell body	CC	4.67	0.0094	0.13	44	44297
Positive regulation of	BP	4.67	0.0094	0.13	13	90277

peptide hormone secretion						
G-protein coupled receptor signaling pathway	BP	4.67	0.0094	0.13	84	7186
Cellular nitrogen compound biosynthetic process	BP	4.66	0.0095	0.13	366	44271
Pancreas development	BP	4.65	0.0095	0.13	7	31016
Ventricular septum morphogenesis	BP	4.65	0.0095	0.13	7	60412
Protein localization	BP	4.65	0.0096	0.13	51	8104
Regulation of actin cytoskeleton organization	BP	4.64	0.0096	0.13	35	32956
Regulation of cyclic nucleotide metabolic process	BP	4.64	0.0096	0.13	25	30799
Binding, bridging	MF	4.64	0.0097	0.13	27	60090
Neuronal cell body	CC	4.64	0.0097	0.13	40	43025
Canonical Wnt receptor	BP	4.62	0.0099	0.13	17	60070

signaling pathway						
Atrial septum morphogenesis	BP	4.61	0.0099	0.13	5	60413
Outer membrane-bounded periplasmic space	CC	4.61	0.0099	0.13	5	30288
Periplasmic space	CC	4.61	0.0099	0.13	5	42597
Detection of bacterium	BP	4.61	0.0099	0.13	5	16045
Endocytic recycling	BP	4.61	0.0099	0.13	5	32456
Microtubule	CC	4.61	0.01	0.13	58	5874
Positive regulation of transport	BP	4.61	0.01	0.13	79	51050
Hepatocyte differentiation	BP	4.59	0.01	0.13	4	70365
Glomerular visceral epithelial cell development	BP	4.59	0.01	0.13	4	72015
Hydrogen peroxide biosynthetic process	BP	4.59	0.01	0.13	4	50665
RNA-induced silencing	CC	4.59	0.01	0.13	4	16442

complex						
Rnai effector complex	CC	4.59	0.01	0.13	4	31332
Regulation of dendritic cell antigen processing and presentation	BP	4.59	0.01	0.13	4	2604
Left/right axis specification	BP	4.59	0.01	0.13	4	70986
CARD domain binding	MF	4.59	0.01	0.13	4	50700
Neuron migration	BP	4.59	0.01	0.13	21	1764
Cellular localization	BP	4.59	0.01	0.13	49	51641
Regulation of cell morphogenesis involved in differentiation	BP	4.59	0.01	0.13	34	10769
Regulation of cellular component movement	BP	4.58	0.01	0.13	73	51270
Glucan metabolic process	BP	4.55	0.01	0.13	12	44042
Cellular glucan metabolic process	BP	4.55	0.01	0.13	12	6073
Brain development	BP	4.55	0.01	0.13	32	7420

Transcription regulatory region DNA binding	MF	4.55	0.01	0.13	58	44212
Cell proliferation	BP	4.55	0.01	0.13	88	8283
Regulation of interleukin-1 beta production	BP	4.53	0.01	0.13	10	32651
Biosynthetic process	BP	4.52	0.01	0.13	507	9058
Cytoplasmic vesicle	CC	4.51	0.01	0.14	97	31410
Positive regulation of peptide secretion	BP	4.51	0.01	0.14	13	2793
Positive regulation of cytokine biosynthetic process	BP	4.49	0.01	0.14	14	42108
Appendage morphogenesis	BP	4.48	0.01	0.14	21	35107
Limb morphogenesis	BP	4.48	0.01	0.14	21	35108
Embryonic forelimb morphogenesis	BP	4.45	0.01	0.14	9	35115
Tight junction assembly	BP	4.45	0.01	0.14	9	70830
Positive regulation of	BP	4.45	0.01	0.14	25	45785

cell adhesion						
Cellular response to acid	BP	4.44	0.01	0.14	11	71229
Golgi membrane	CC	4.43	0.01	0.14	80	139
Asymmetric protein localization	BP	4.42	0.01	0.14	6	8105
Positive regulation of developmental process	BP	4.42	0.01	0.14	106	51094
Neuron development	BP	4.41	0.01	0.15	22	48666
Potassium channel activity	MF	4.41	0.01	0.15	22	5267
Regulation of camp metabolic process	BP	4.41	0.01	0.15	22	30814
Cytoplasmic part	CC	4.40	0.01	0.15	888	44444
Intrinsic to plasma membrane	CC	4.40	0.01	0.15	156	31226
Coated vesicle	CC	4.40	0.01	0.15	26	30135
Clathrin-coated vesicle	CC	4.40	0.01	0.15	24	30136
Negative regulation of developmental growth	BP	4.40	0.01	0.15	8	48640
Insulin-like growth	MF	4.40	0.01	0.15	8	5520

factor binding						
Regulation of locomotion	BP	4.39	0.01	0.15	73	40012
Intracellular organelle	CC	4.39	0.01	0.15	1114	43229
Cellular response to vascular endothelial growth factor stimulus	BP	4.38	0.01	0.15	7	35924
Positive regulation of epithelial to mesenchymal transition	BP	4.38	0.01	0.15	7	10718
DNA-dependent transcription, initiation	BP	4.37	0.01	0.15	38	6352
Calcium channel activity	MF	4.37	0.01	0.15	21	5262
Lung development	BP	4.36	0.01	0.15	17	30324
Regulation of gliogenesis	BP	4.34	0.01	0.15	14	14013
Regulation of axon extension	BP	4.34	0.01	0.15	10	30516
Positive regulation of cyclase activity	BP	4.34	0.01	0.15	10	31281

Aromatic compound biosynthetic process	BP	4.34	0.01	0.15	355	19438
Organic cyclic compound biosynthetic process	BP	4.32	0.01	0.15	370	1901362
Organelle lumen	CC	4.32	0.01	0.15	90	43233
Cellular_component	CC	4.30	0.01	0.15	2166	5575
Aminoglycan metabolic process	BP	4.30	0.01	0.15	29	6022
Digestive tract development	BP	4.27	0.01	0.16	11	48565
Cortical cytoskeleton	CC	4.27	0.01	0.16	11	30863
Inorganic cation transmembrane transporter activity	MF	4.26	0.01	0.16	74	22890
Anterior/posterior axis specification	BP	4.25	0.01	0.16	9	9948
Cytoskeleton	CC	4.23	0.01	0.16	106	5856
Cellular membrane organization	BP	4.23	0.01	0.16	64	16044

Plasma membrane	CC	4.23	0.01	0.16	488	5886
Dopaminergic neuron differentiation	BP	4.23	0.01	0.16	5	71542
Regulation of respiratory gaseous exchange by neurological system process	BP	4.23	0.01	0.16	5	2087
Positive regulation of Wnt receptor signaling pathway	BP	4.22	0.01	0.16	16	30177
Phosphoglucomutase activity	MF	4.22	0.01	0.16	3	4614
Microvillus assembly	BP	4.22	0.01	0.16	3	30033
Microvillus organization	BP	4.22	0.01	0.16	3	32528
Noradrenergic neuron differentiation	BP	4.22	0.01	0.16	3	3357
Positive regulation of Wnt receptor signaling pathway, planar cell polarity pathway	BP	4.22	0.01	0.16	3	2000096
Response to oleic acid	BP	4.22	0.01	0.16	3	34201

Chemorepellent activity	MF	4.22	0.01	0.16	3	45499
Positive regulation of steroid hormone biosynthetic process	BP	4.22	0.01	0.16	3	90031
Membrane-bounded organelle	CC	4.19	0.02	0.17	1000	43227
Generation of precursor metabolites and energy	BP	4.17	0.02	0.17	63	6091
Non-canonical Wnt receptor signaling pathway	BP	4.17	0.02	0.17	8	35567
Microtubule-based movement	BP	4.16	0.02	0.17	28	7018
Heterocycle biosynthetic process	BP	4.16	0.02	0.17	354	18130
Nucleobase-containing compound biosynthetic process	BP	4.15	0.02	0.17	345	34654
Glycogen biosynthetic process	BP	4.13	0.02	0.17	6	5978
Glucan biosynthetic	BP	4.13	0.02	0.17	6	9250

process						
Cell differentiation in hindbrain	BP	4.13	0.02	0.17	6	21533
MHC class II protein complex	CC	4.13	0.02	0.17	6	42613
Regulation of timing of cell differentiation	BP	4.11	0.02	0.17	4	48505
Glomerular epithelial cell development	BP	4.11	0.02	0.17	4	72310
Negative regulation of catenin import into nucleus	BP	4.11	0.02	0.17	4	35414
Negative regulation of smooth muscle cell differentiation	BP	4.11	0.02	0.17	4	51151
Pinocytosis	BP	4.11	0.02	0.17	4	6907
Semaphorin receptor activity	MF	4.11	0.02	0.17	4	17154
RNA polymerase II core binding	MF	4.11	0.02	0.17	4	993
Regulation of glial cell	BP	4.10	0.02	0.17	11	45685

differentiation						
Regulation of stem cell proliferation	BP	4.10	0.02	0.18	16	72091
Extracellular matrix organization	BP	4.09	0.02	0.18	43	30198
Regulation of DNA binding	BP	4.08	0.02	0.18	15	51101
Organic cyclic compound binding	MF	4.07	0.02	0.18	721	97159
Regulation of chemokine production	BP	4.07	0.02	0.18	12	32642
Phagocytic vesicle membrane	CC	4.07	0.02	0.18	12	30670
Palate development	BP	4.06	0.02	0.18	14	60021
Regulation of cyclase activity	BP	4.06	0.02	0.18	14	31279
Cellular biosynthetic process	BP	4.06	0.02	0.18	482	44249
Positive regulation of gliogenesis	BP	4.05	0.02	0.18	9	14015

Tissue homeostasis	BP	4.04	0.02	0.18	18	1894
Regulation of cell projection organization	BP	4.03	0.02	0.18	44	31344
Extracellular structure organization	BP	4.02	0.02	0.18	43	43062
Actin binding	MF	4.01	0.02	0.19	58	3779
Endosome membrane	CC	4.00	0.02	0.19	51	10008
Regulation of cell growth	BP	3.98	0.02	0.19	48	1558
Positive regulation of lyase activity	BP	3.98	0.02	0.19	10	51349
Response to fatty acid	BP	3.98	0.02	0.19	10	70542
Regulation of epithelial to mesenchymal transition	BP	3.98	0.02	0.19	10	10717
Tube morphogenesis	BP	3.97	0.02	0.19	19	35239
Macromolecule biosynthetic process	BP	3.97	0.02	0.19	384	9059
Embryo development ending in birth or egg	BP	3.97	0.02	0.19	37	9792

hatching						
Growth factor binding	MF	3.96	0.02	0.19	21	19838
Antigen processing and presentation of exogenous peptide antigen	BP	3.96	0.02	0.19	30	2478
Positive regulation of phagocytosis	BP	3.96	0.02	0.19	8	50766
Vesicle-mediated transport	BP	3.94	0.02	0.19	112	16192
Coated pit	CC	3.94	0.02	0.19	11	5905
Membrane organization	BP	3.94	0.02	0.19	64	61024
Glycosaminoglycan metabolic process	BP	3.94	0.02	0.19	27	30203
Divalent inorganic cation transmembrane transporter activity	MF	3.94	0.02	0.19	27	72509
Protein localization to membrane	BP	3.93	0.02	0.19	14	72657
Heart looping	BP	3.92	0.02	0.20	12	1947

Cell morphogenesis involved in differentiation	BP	3.92	0.02	0.20	22	904
Anchoring junction	CC	3.90	0.02	0.20	37	70161
Regulation of transcription involved in cell fate commitment	BP	3.89	0.02	0.20	5	60850
Spinal cord association neuron differentiation	BP	3.89	0.02	0.20	5	21527
Intrinsic to internal side of plasma membrane	CC	3.89	0.02	0.20	5	31235
Dorsal/ventral axis specification	BP	3.89	0.02	0.20	5	9950
Regulation of respiratory system process	BP	3.89	0.02	0.20	5	44065
Lens morphogenesis in camera-type eye	BP	3.89	0.02	0.20	5	2089
DNA-directed RNA polymerase II, core complex	CC	3.89	0.02	0.20	5	5665
Actin monomer binding	MF	3.89	0.02	0.20	5	3785

Central nervous system neuron development	BP	3.89	0.02	0.20	7	21954
Mesenchymal cell development	BP	3.89	0.02	0.20	7	14031
Positive regulation of glial cell differentiation	BP	3.89	0.02	0.20	7	45687
Zinc ion binding	MF	3.88	0.02	0.20	280	8270
Positive regulation of adenylate cyclase activity	BP	3.86	0.02	0.20	9	45762
Endosomal part	CC	3.86	0.02	0.20	53	44440
Neurotransmitter:sodium symporter activity	MF	3.85	0.02	0.20	6	5328
Regulation of transcription regulatory region DNA binding	BP	3.85	0.02	0.20	6	2000677
Cytoskeletal protein binding	MF	3.85	0.02	0.20	97	8092
Regulation of cellular biosynthetic process	BP	3.84	0.02	0.20	458	31326
Regulation of molecular	BP	3.84	0.02	0.21	254	65009

function						
Anion transmembrane transporter activity	MF	3.83	0.02	0.21	40	8509
Polysaccharide metabolic process	BP	3.83	0.02	0.21	15	5976
Regulation of stem cell differentiation	BP	3.83	0.02	0.21	15	2000736
Anatomical structure homeostasis	BP	3.82	0.02	0.21	29	60249
Regulation of interleukin-1 production	BP	3.81	0.02	0.21	10	32652
Stem cell proliferation	BP	3.81	0.02	0.21	10	72089
Antigen processing and presentation of exogenous antigen	BP	3.81	0.02	0.21	30	19884
Myosin complex	CC	3.80	0.02	0.21	14	16459
Membrane part	CC	3.80	0.02	0.21	808	44425
Voltage-gated channel activity	MF	3.80	0.02	0.21	31	22832
Voltage-gated ion	MF	3.80	0.02	0.21	31	5244

channel activity						
Regulation of adenylate cyclase activity	BP	3.79	0.02	0.21	13	45761
Regulation of cartilage development	BP	3.79	0.02	0.21	11	61035
Positive regulation of lipase activity	BP	3.78	0.02	0.21	17	60193
Organelle membrane	CC	3.78	0.02	0.21	307	31090
Positive regulation of JNK cascade	BP	3.78	0.02	0.21	12	46330
Regulation of cell motility	BP	3.78	0.02	0.21	66	2000145
Synapse	CC	3.78	0.02	0.21	34	45202
Positive regulation of cytoskeleton organization	BP	3.77	0.02	0.21	21	51495
Regulation of neuron projection development	BP	3.77	0.02	0.21	36	10975
Cell part morphogenesis	BP	3.77	0.02	0.21	38	32990
Cell differentiation in	BP	3.76	0.02	0.21	8	21515

spinal cord						
Face morphogenesis	BP	3.76	0.02	0.21	8	60325
Stem cell development	BP	3.76	0.02	0.21	8	48864
Activation of adenylate cyclase activity	BP	3.76	0.02	0.21	8	7190
Embryo development	BP	3.74	0.02	0.21	52	9790
Heterocyclic compound binding	MF	3.72	0.02	0.22	709	1901363
Regulation of macromolecule metabolic process	BP	3.72	0.02	0.22	565	60255
Stress-activated MAPK cascade	BP	3.72	0.02	0.22	18	51403
Regulation of RNA metabolic process	BP	3.71	0.02	0.22	406	51252
Hexose metabolic process	BP	3.71	0.02	0.22	33	19318
Cell projection organization	BP	3.70	0.02	0.22	75	30030
Positive regulation of chemokine biosynthetic	BP	3.70	0.02	0.22	4	45080

process						
Hematopoietic stem cell proliferation	BP	3.70	0.02	0.22	4	71425
Regulation of development, heterochronic	BP	3.70	0.02	0.22	4	40034
Apical protein localization	BP	3.70	0.02	0.22	4	45176
Ventricular trabecula myocardium morphogenesis	BP	3.70	0.02	0.22	4	3222
Positive regulation of protein sumoylation	BP	3.70	0.02	0.22	4	33235
Pre-mrna binding	MF	3.70	0.02	0.22	4	36002
Peripheral nervous system neuron development	BP	3.70	0.02	0.22	4	48935
Metaphase	BP	3.70	0.02	0.22	4	51323
Regulation of superoxide anion generation	BP	3.70	0.02	0.22	4	32928

Activation of transmembrane receptor protein tyrosine kinase activity	BP	3.70	0.02	0.22	4	7171
DNA synthesis involved in DNA repair	BP	3.70	0.02	0.22	4	731
Positive regulation of SMAD protein import into nucleus	BP	3.70	0.02	0.22	4	60391
Basal transcription machinery binding	MF	3.70	0.02	0.22	4	1098
Basal RNA polymerase II transcription machinery binding	MF	3.70	0.02	0.22	4	1099
Protein domain specific binding	MF	3.69	0.03	0.22	79	19904
Cellular response to endogenous stimulus	BP	3.69	0.03	0.22	100	71495
Heterotrimeric G-protein complex	CC	3.69	0.03	0.22	9	5834
Regulation of lyase	BP	3.68	0.03	0.22	14	51339

activity						
Amino acid transmembrane transporter activity	MF	3.68	0.03	0.22	14	15171
Voltage-gated cation channel activity	MF	3.68	0.03	0.22	24	22843
Axon part	CC	3.68	0.03	0.22	24	33267
Positive regulation of protein catabolic process	BP	3.68	0.03	0.22	17	45732
Endoderm development	BP	3.67	0.03	0.22	7	7492
Chondroitin sulfate biosynthetic process	BP	3.67	0.03	0.22	7	30206
Dorsal/ventral pattern formation	BP	3.66	0.03	0.22	13	9953
Positive regulation of cell projection organization	BP	3.65	0.03	0.22	25	31346
Recycling endosome	CC	3.64	0.03	0.22	12	55037
Response to organic substance	BP	3.64	0.03	0.22	261	10033

Voltage-gated potassium channel activity	MF	3.63	0.03	0.22	16	5249
Regulation of biosynthetic process	BP	3.63	0.03	0.22	460	9889
Regulation of chemotaxis	BP	3.63	0.03	0.22	20	50920
Response to acid	BP	3.62	0.03	0.22	18	1101
Wnt receptor signaling pathway, calcium modulating pathway	BP	3.62	0.03	0.22	3	7223
Adenylate cyclase binding	MF	3.62	0.03	0.22	3	8179
Regulation of bile acid biosynthetic process	BP	3.62	0.03	0.22	3	70857
Neutrophil degranulation	BP	3.62	0.03	0.22	3	43312
Coumarin metabolic process	BP	3.62	0.03	0.22	3	9804
N-methyl-D-aspartate selective glutamate receptor activity	MF	3.62	0.03	0.22	3	4972

FACIT collagen	CC	3.62	0.03	0.22	3	5593
WINAC complex	CC	3.62	0.03	0.22	3	71778
COPI-coated vesicle	CC	3.62	0.03	0.22	3	30137
Eukaryotic initiation factor 4E binding	MF	3.62	0.03	0.22	3	8190
Opioid receptor signaling pathway	BP	3.62	0.03	0.22	3	38003
Nerve growth factor binding	MF	3.62	0.03	0.22	3	48406
Somatic muscle development	BP	3.62	0.03	0.22	3	7525
Positive regulation of non-canonical Wnt receptor signaling pathway	BP	3.62	0.03	0.22	3	2000052
Positive regulation of sequestering of triglyceride	BP	3.62	0.03	0.22	3	10890
Coronary vasculature morphogenesis	BP	3.62	0.03	0.22	3	60977
Forebrain	BP	3.62	0.03	0.22	3	21797

anterior/posterior pattern specification						
Glycine transmembrane transporter activity	MF	3.62	0.03	0.22	3	15187
Laminin-1 binding	MF	3.62	0.03	0.22	3	43237
Growth plate cartilage development	BP	3.62	0.03	0.22	3	3417
Gene silencing by mirna	BP	3.62	0.03	0.22	3	35195
Retina vasculature morphogenesis in camera-type eye	BP	3.62	0.03	0.22	3	61299
Immunoglobulin production	BP	3.62	0.03	0.22	3	2377
JUN kinase binding	MF	3.62	0.03	0.22	3	8432
Mitotic metaphase	BP	3.62	0.03	0.22	3	89
Sensory perception of sweet taste	BP	3.62	0.03	0.22	3	50916
Chloride channel regulator activity	MF	3.62	0.03	0.22	3	17081
Collagen biosynthetic	BP	3.62	0.03	0.22	3	32964

process						
Endothelial cell differentiation	BP	3.60	0.03	0.22	6	45446
Activation of Rho gtpase activity	BP	3.60	0.03	0.22	6	32862
Intracellular ligand-gated ion channel activity	MF	3.60	0.03	0.22	6	5217
Microfilament motor activity	MF	3.60	0.03	0.22	6	146
Positive regulation of endocytosis	BP	3.59	0.03	0.22	15	45807
Carbohydrate homeostasis	BP	3.59	0.03	0.22	21	33500
Glucose homeostasis	BP	3.59	0.03	0.22	21	42593
Response to arsenic-containing substance	BP	3.59	0.03	0.22	5	46685
Alpha-mannosidase activity	MF	3.59	0.03	0.22	5	4559
Nua4 histone acetyltransferase	CC	3.59	0.03	0.22	5	35267

complex						
Axonemal dynein complex	CC	3.59	0.03	0.22	5	5858
Tubulin-tyrosine ligase activity	MF	3.59	0.03	0.22	5	4835
Extracellular region part	CC	3.58	0.03	0.22	160	44421
Negative regulation of cell growth	BP	3.57	0.03	0.22	25	30308
Regulation of lipid biosynthetic process	BP	3.57	0.03	0.22	19	46890
Tissue remodeling	BP	3.56	0.03	0.23	14	48771
Regulation of cell morphogenesis	BP	3.56	0.03	0.23	49	22604
Intracellular membrane-bounded organelle	CC	3.54	0.03	0.23	990	43231
Potassium ion transmembrane transporter activity	MF	3.53	0.03	0.23	23	15079
Metalloendopeptidase activity	MF	3.53	0.03	0.23	20	4222

Negative regulation of cell projection organization	BP	3.53	0.03	0.23	16	31345
Regulation of exocytosis	BP	3.53	0.03	0.23	16	17157
Endocytic vesicle	CC	3.53	0.03	0.23	16	30139
Stress-activated protein kinase signaling cascade	BP	3.52	0.03	0.23	18	31098
Adenyl ribonucleotide binding	MF	3.52	0.03	0.23	212	32559
Cellular response to amino acid stimulus	BP	3.52	0.03	0.23	9	71230
In utero embryonic development	BP	3.51	0.03	0.23	35	1701
Phosphotransferase activity, alcohol group as acceptor	MF	3.51	0.03	0.23	107	16773
Intracellular organelle lumen	CC	3.51	0.03	0.23	80	70013
Neurotrophin TRK receptor signaling pathway	BP	3.51	0.03	0.23	44	48011

Embryonic organ development	BP	3.50	0.03	0.23	21	48568
Regulation of lipase activity	BP	3.50	0.03	0.23	21	60191
Skin development	BP	3.50	0.03	0.23	10	43588
Amide biosynthetic process	BP	3.50	0.03	0.23	11	43604
Protein kinase activity	MF	3.48	0.03	0.24	91	4672
Regulation of cell-cell adhesion	BP	3.48	0.03	0.24	15	22407
Epithelial cell differentiation	BP	3.48	0.03	0.24	41	30855
Embryonic limb morphogenesis	BP	3.47	0.03	0.24	17	30326
Embryonic appendage morphogenesis	BP	3.47	0.03	0.24	17	35113
ATP binding	MF	3.47	0.03	0.24	208	5524
Negative regulation of intracellular steroid hormone receptor signaling pathway	BP	3.46	0.03	0.24	7	33144

Vesicle docking involved in exocytosis	BP	3.46	0.03	0.24	7	6904
Glutamate receptor activity	MF	3.46	0.03	0.24	7	8066
Ephrin receptor signaling pathway	BP	3.46	0.03	0.24	7	48013
Negative regulation of stress-activated MAPK cascade	BP	3.46	0.03	0.24	7	32873
Negative regulation of stress-activated protein kinase signaling cascade	BP	3.46	0.03	0.24	7	70303
Neurotrophin signaling pathway	BP	3.45	0.03	0.24	44	38179
Transporter activity	MF	3.43	0.03	0.24	170	5215
Monosaccharide catabolic process	BP	3.42	0.03	0.25	16	46365
Notch signaling pathway	BP	3.41	0.03	0.25	21	7219
Positive regulation of stress-activated MAPK cascade	BP	3.41	0.03	0.25	13	32874

Positive regulation of cell-substrate adhesion	BP	3.41	0.03	0.25	13	10811
Organelle localization	BP	3.41	0.03	0.25	13	51640
Lysosomal membrane	CC	3.41	0.03	0.25	26	5765
Adenyl nucleotide binding	MF	3.40	0.03	0.25	212	30554
Proteinaceous extracellular matrix	CC	3.39	0.03	0.25	36	5578
Calcium ion transmembrane transporter activity	MF	3.39	0.03	0.25	22	15085
Chordate embryonic development	BP	3.39	0.03	0.25	35	43009
Endoplasmic reticulum lumen	CC	3.39	0.03	0.25	29	5788
Regulation of Ras gtpase activity	BP	3.39	0.03	0.25	29	32318
Antigen processing and presentation of peptide antigen	BP	3.38	0.03	0.25	31	48002
Response to amino acid	BP	3.37	0.03	0.25	15	43200

stimulus						
Positive regulation of phospholipase activity	BP	3.37	0.03	0.25	15	10518
Transition metal ion binding	MF	3.37	0.03	0.25	308	46914
Negative regulation of response to external stimulus	BP	3.37	0.03	0.25	23	32102
Regulation of gene expression	BP	3.37	0.03	0.25	455	10468
Regulation of embryonic development	BP	3.37	0.03	0.25	17	45995
Macromolecule metabolic process	BP	3.37	0.03	0.25	834	43170
Cellular macromolecule biosynthetic process	BP	3.37	0.03	0.25	355	34645
Polysaccharide catabolic process	BP	3.37	0.03	0.25	6	272
Adipose tissue development	BP	3.37	0.03	0.25	6	60612
Cardiac cell	BP	3.37	0.03	0.25	6	55006

development						
Homeostasis of number of cells within a tissue	BP	3.37	0.03	0.25	6	48873
Negative regulation of protein kinase B signaling cascade	BP	3.37	0.03	0.25	6	51898
Regulation of interleukin-4 production	BP	3.37	0.03	0.25	6	32673
Regulation of RNA biosynthetic process	BP	3.36	0.03	0.25	395	2001141
Negative regulation of muscle cell differentiation	BP	3.36	0.03	0.25	9	51148
Negative regulation of ERBB signaling pathway	BP	3.36	0.03	0.25	9	1901185
Negative regulation of epidermal growth factor receptor signaling pathway	BP	3.36	0.03	0.25	9	42059
Positive regulation of proteasomal protein	BP	3.36	0.03	0.25	9	1901800

catabolic process						
Integral to plasma membrane	CC	3.35	0.04	0.25	145	5887
MHC protein complex	CC	3.35	0.04	0.25	10	42611
RNA polymerase activity	MF	3.35	0.04	0.25	10	34062
DNA-directed RNA polymerase activity	MF	3.35	0.04	0.25	10	3899
Regulation of organ morphogenesis	BP	3.34	0.04	0.25	25	2000027
Glandular epithelial cell development	BP	3.34	0.04	0.25	4	2068
Negative chemotaxis	BP	3.34	0.04	0.25	4	50919
Intramolecular transferase activity, phosphotransferases	MF	3.34	0.04	0.25	4	16868
Positive regulation of translational initiation	BP	3.34	0.04	0.25	4	45948
Platelet alpha granule	CC	3.34	0.04	0.25	4	31091
Heart trabecula	BP	3.34	0.04	0.25	4	61384

morphogenesis						
Myelin maintenance	BP	3.34	0.04	0.25	4	43217
Regulation of Wnt receptor signaling pathway, planar cell polarity pathway	BP	3.34	0.04	0.25	4	2000095
Regulation of antigen processing and presentation	BP	3.34	0.04	0.25	4	2577
Dipeptidyl-peptidase activity	MF	3.34	0.04	0.25	4	8239
Positive regulation of steroid biosynthetic process	BP	3.34	0.04	0.25	4	10893
RNA polymerase core enzyme binding	MF	3.34	0.04	0.25	4	43175
Phosphorylation	BP	3.34	0.04	0.25	96	16310
Positive regulation of organ growth	BP	3.32	0.04	0.25	5	46622
Translation initiation factor binding	MF	3.32	0.04	0.25	5	31369

Nitric oxide mediated signal transduction	BP	3.32	0.04	0.25	5	7263
Organ formation	BP	3.32	0.04	0.25	5	48645
Vitamin D receptor binding	MF	3.32	0.04	0.25	5	42809
H4/H2A histone acetyltransferase complex	CC	3.32	0.04	0.25	5	43189
Negative regulation of toll-like receptor signaling pathway	BP	3.32	0.04	0.25	5	34122
Nucleotide-sugar biosynthetic process	BP	3.32	0.04	0.25	5	9226
Regulation of cellular macromolecule biosynthetic process	BP	3.31	0.04	0.25	426	2000112
Calcium ion binding	MF	3.29	0.04	0.25	99	5509
Positive regulation of stress-activated protein kinase signaling cascade	BP	3.29	0.04	0.25	13	70304
Positive regulation of	BP	3.29	0.04	0.25	13	10863

phospholipase C activity						
Monosaccharide metabolic process	BP	3.27	0.04	0.25	36	5996
Basement membrane	CC	3.27	0.04	0.25	15	5604
Hexose catabolic process	BP	3.27	0.04	0.25	15	19320
Positive regulation of neural precursor cell proliferation	BP	3.27	0.04	0.25	7	2000179
Substrate adhesion-dependent cell spreading	BP	3.27	0.04	0.25	7	34446
Calcium-dependent cell-cell adhesion	BP	3.27	0.04	0.25	7	16339
Enhancer sequence-specific DNA binding	MF	3.27	0.04	0.25	7	1158
Regulated secretory pathway	BP	3.27	0.04	0.25	7	45055
Autophagic vacuole	CC	3.27	0.04	0.25	7	5776
Positive regulation of stem cell proliferation	BP	3.26	0.04	0.25	12	2000648

Activation of phospholipase C activity	BP	3.26	0.04	0.25	12	7202
Chondroitin sulfate metabolic process	BP	3.26	0.04	0.25	12	30204
Calmodulin binding	MF	3.25	0.04	0.25	28	5516
Positive regulation of interleukin-6 production	BP	3.22	0.04	0.25	8	32755
Regulation of actin filament depolymerization	BP	3.22	0.04	0.25	8	30834
Negative regulation of cell-substrate adhesion	BP	3.22	0.04	0.25	8	10812
Cell projection morphogenesis	BP	3.21	0.04	0.25	35	48858
Actin cytoskeleton reorganization	BP	3.20	0.04	0.25	9	31532
Extracellular matrix binding	MF	3.20	0.04	0.25	9	50840
Ammonium transmembrane transport	BP	3.20	0.04	0.25	2	72488
Prostaglandin-E	MF	3.20	0.04	0.25	2	50220

synthase activity						
Trehalose metabolic process	BP	3.20	0.04	0.25	2	5991
Seminiferous tubule development	BP	3.20	0.04	0.25	2	72520
Cardiac atrium morphogenesis	BP	3.20	0.04	0.25	2	3209
Regulation of collateral sprouting in absence of injury	BP	3.20	0.04	0.25	2	48696
Spemann organizer formation	BP	3.20	0.04	0.25	2	60061
Regulation of exocyst localization	BP	3.20	0.04	0.25	2	60178
Dehydroascorbic acid transporter activity	MF	3.20	0.04	0.25	2	33300
D-glucose transmembrane transporter activity	MF	3.20	0.04	0.25	2	55056
Production of mirnas involved in gene	BP	3.20	0.04	0.25	2	35196

silencing by mirna						
Melatonin receptor activity	MF	3.20	0.04	0.25	2	8502
Hypothalamus cell differentiation	BP	3.20	0.04	0.25	2	21979
Collagen type IX	CC	3.20	0.04	0.25	2	5594
Negative regulation of transcription by transcription factor localization	BP	3.20	0.04	0.25	2	10621
Detection of endogenous stimulus	BP	3.20	0.04	0.25	2	9726
Autonomic nervous system development	BP	3.20	0.04	0.25	2	48483
Trophectodermal cellular morphogenesis	BP	3.20	0.04	0.25	2	1831
Sec61 translocon complex	CC	3.20	0.04	0.25	2	5784
Translocon complex	CC	3.20	0.04	0.25	2	71256
Brain segmentation	BP	3.20	0.04	0.25	2	35284

Cell migration involved in endocardial cushion formation	BP	3.20	0.04	0.25	2	3273
Transcription initiation from RNA polymerase III promoter	BP	3.20	0.04	0.25	2	6384
Calcium ion-dependent exocytosis of neurotransmitter	BP	3.20	0.04	0.25	2	48791
Dendritic growth cone	CC	3.20	0.04	0.25	2	44294
Natriuretic peptide receptor activity	MF	3.20	0.04	0.25	2	16941
Metencephalon development	BP	3.20	0.04	0.25	2	22037
Diencephalon morphogenesis	BP	3.20	0.04	0.25	2	48852
Cardiac vascular smooth muscle cell development	BP	3.20	0.04	0.25	2	60948
Vasoactive intestinal polypeptide receptor activity	MF	3.20	0.04	0.25	2	4999

Transcription initiation from mitochondrial promoter	BP	3.20	0.04	0.25	2	6391
G-quadruplex DNA binding	MF	3.20	0.04	0.25	2	51880
Butyrate metabolic process	BP	3.20	0.04	0.25	2	19605
Chromosome movement towards spindle pole	BP	3.20	0.04	0.25	2	51305
Phenylpropanoid catabolic process	BP	3.20	0.04	0.25	2	46271
Cerebral cortex regionalization	BP	3.20	0.04	0.25	2	21796
Regulation of defense response to bacterium	BP	3.20	0.04	0.25	2	1900424
Regulation of phagocytosis, engulfment	BP	3.20	0.04	0.25	2	60099
Positive regulation of phagocytosis, engulfment	BP	3.20	0.04	0.25	2	60100

Cellular pigmentation	BP	3.20	0.04	0.25	2	33059
Synaptic cleft	CC	3.20	0.04	0.25	2	43083
Regulation of nodal signaling pathway involved in determination of left/right asymmetry	BP	3.20	0.04	0.25	2	1900145
Regulation of nodal signaling pathway involved in determination of lateral mesoderm left/right asymmetry	BP	3.20	0.04	0.25	2	1900175
Establishment or maintenance of neuroblast polarity	BP	3.20	0.04	0.25	2	45196
Establishment of neuroblast polarity	BP	3.20	0.04	0.25	2	45200
Sensory system development	BP	3.20	0.04	0.25	2	48880
Cardiac muscle cell myoblast differentiation	BP	3.20	0.04	0.25	2	60379

Collagen V binding	MF	3.20	0.04	0.25	2	70052
Forebrain neuron fate commitment	BP	3.20	0.04	0.25	2	21877
Regulation of transcription from RNA polymerase II promoter involved in spinal cord motor neuron fate specification	BP	3.20	0.04	0.25	2	21912
Neural plate pattern specification	BP	3.20	0.04	0.25	2	60896
Neural plate regionalization	BP	3.20	0.04	0.25	2	60897
Synaptic vesicle clustering	BP	3.20	0.04	0.25	2	97091
Bombesin receptor activity	MF	3.20	0.04	0.25	2	4946
Locomotion involved in locomotory behavior	BP	3.20	0.04	0.25	2	31987
Regulation of transcription from RNA polymerase II promoter	BP	3.20	0.04	0.25	2	1900094

involved in determination of left/right symmetry						
Nodal signaling pathway involved in determination of lateral mesoderm left/right asymmetry	BP	3.20	0.04	0.25	2	1900164
Nodal signaling pathway involved in determination of left/right asymmetry	BP	3.20	0.04	0.25	2	38107
Smooth muscle cell proliferation	BP	3.20	0.04	0.25	2	48659
Pre-mrna intronic binding	MF	3.20	0.04	0.25	2	97157
Intestinal epithelial cell development	BP	3.20	0.04	0.25	2	60576
Negative regulation of cholesterol biosynthetic process	BP	3.20	0.04	0.25	2	45541
Negative regulation of	BP	3.20	0.04	0.25	2	90206

cholesterol metabolic process						
Actin polymerization-dependent cell motility	BP	3.20	0.04	0.25	2	70358
Protein poly-ADP-ribosylation	BP	3.20	0.04	0.25	2	70212
Viral assembly, maturation, egress, and release	BP	3.20	0.04	0.25	2	19067
Retinal blood vessel morphogenesis	BP	3.20	0.04	0.25	2	61304
Ornithine decarboxylase activator activity	MF	3.20	0.04	0.25	2	42978
Intestine smooth muscle contraction	BP	3.20	0.04	0.25	2	14827
Calcium-dependent protein kinase C activity	MF	3.20	0.04	0.25	2	4698
Calcium-dependent protein serine/threonine kinase activity	MF	3.20	0.04	0.25	2	9931
Laminin-11 complex	CC	3.20	0.04	0.25	2	43260

Positive regulation of hydrolase activity	BP	3.19	0.04	0.25	69	51345
Regulation of MAPK cascade	BP	3.19	0.04	0.25	71	43408
Ubiquitin protein ligase binding	MF	3.19	0.04	0.25	26	31625
Small conjugating protein ligase binding	MF	3.19	0.04	0.25	26	44389
Transcription initiation from RNA polymerase II promoter	BP	3.19	0.04	0.25	31	6367
Regulation of purine nucleotide biosynthetic process	BP	3.18	0.04	0.25	20	1900371
Regulation of nucleotide biosynthetic process	BP	3.18	0.04	0.25	20	30808
Transcriptional repressor complex	CC	3.18	0.04	0.25	13	17053
Ion transport	BP	3.17	0.04	0.25	130	6811
Regulation of transcription, DNA-	BP	3.17	0.04	0.25	391	6355

dependent						
Positive regulation of purine nucleotide metabolic process	BP	3.17	0.04	0.25	15	1900544
Negative regulation of binding	BP	3.17	0.04	0.25	15	51100
Wnt receptor signaling pathway, planar cell polarity pathway	BP	3.16	0.04	0.25	6	60071
Regulation of establishment of planar polarity	BP	3.16	0.04	0.25	6	90175
Negative regulation of glial cell differentiation	BP	3.16	0.04	0.25	6	45686
Motor neuron axon guidance	BP	3.16	0.04	0.25	6	8045
Laminin binding	MF	3.16	0.04	0.25	6	43236
Regulation of cytokine secretion involved in immune response	BP	3.15	0.04	0.25	3	2739
Galactose catabolic	BP	3.15	0.04	0.25	3	19388

process						
Filtration diaphragm	CC	3.15	0.04	0.25	3	36056
Slit diaphragm	CC	3.15	0.04	0.25	3	36057
Relaxation of smooth muscle	BP	3.15	0.04	0.25	3	44557
Embryonic process involved in female pregnancy	BP	3.15	0.04	0.25	3	60136
Regulation of neuron migration	BP	3.15	0.04	0.25	3	2001222
Cellular extravasation	BP	3.15	0.04	0.25	3	45123
Interkinetic nuclear migration	BP	3.15	0.04	0.25	3	22027
CD4-positive or CD8-positive, alpha-beta T cell lineage commitment	BP	3.15	0.04	0.25	3	43369
H3 histone acetyltransferase complex	CC	3.15	0.04	0.25	3	70775
MOZ/MORF histone acetyltransferase	CC	3.15	0.04	0.25	3	70776

complex						
Trna transcription	BP	3.15	0.04	0.25	3	9304
Neuronal cell body membrane	CC	3.15	0.04	0.25	3	32809
Cell body membrane	CC	3.15	0.04	0.25	3	44298
MAP-kinase scaffold activity	MF	3.15	0.04	0.25	3	5078
Positive regulation of dendritic cell antigen processing and presentation	BP	3.15	0.04	0.25	3	2606
Nuclear envelope lumen	CC	3.15	0.04	0.25	3	5641
Laminin-1 complex	CC	3.15	0.04	0.25	3	5606
Cell cortex part	CC	3.15	0.04	0.25	18	44448
Enzyme activator activity	MF	3.15	0.04	0.25	64	8047
Protein secretion	BP	3.14	0.04	0.26	12	9306
Regulation of cell migration	BP	3.13	0.04	0.26	61	30334
Regulation of	BP	3.12	0.04	0.26	16	1505

neurotransmitter levels						
Visual perception	BP	3.12	0.04	0.26	30	7601
Dendrite	CC	3.12	0.04	0.26	30	30425
Detection of external stimulus	BP	3.12	0.04	0.26	26	9581
Monocarboxylic acid biosynthetic process	BP	3.12	0.04	0.26	28	72330
Central nervous system development	BP	3.10	0.04	0.26	20	7417
Activating transcription factor binding	MF	3.10	0.04	0.26	11	33613
Negative regulation of cellular response to growth factor stimulus	BP	3.09	0.05	0.27	17	90288
G-protein coupled peptide receptor activity	MF	3.09	0.05	0.27	21	8528
E-box binding	MF	3.08	0.05	0.27	7	70888
Myeloid cell activation involved in immune response	BP	3.08	0.05	0.27	7	2275

Vesicle docking	BP	3.08	0.05	0.27	7	48278
Excretion	BP	3.07	0.05	0.27	10	7588
Negative regulation of steroid biosynthetic process	BP	3.07	0.05	0.27	5	10894
SMAD protein signal transduction	BP	3.07	0.05	0.27	5	60395
External encapsulating structure part	CC	3.07	0.05	0.27	5	44462
RNA polymerase II distal enhancer sequence-specific DNA binding	MF	3.07	0.05	0.27	5	980
Mannosidase activity	MF	3.07	0.05	0.27	5	15923
Positive regulation of neuroblast proliferation	BP	3.07	0.05	0.27	5	2052
Protein phosphatase 2A binding	MF	3.07	0.05	0.27	5	51721
Histone acetyltransferase binding	MF	3.07	0.05	0.27	5	35035
Regulation of alpha-	BP	3.07	0.05	0.27	5	2000311

amino-3-hydroxy-5-methyl-4-isoxazole propionate selective glutamate receptor activity						
Protein targeting to plasma membrane	BP	3.07	0.05	0.27	5	72661
Muscle cell proliferation	BP	3.07	0.05	0.27	5	33002
Positive regulation of nucleotide metabolic process	BP	3.07	0.05	0.27	15	45981
Cellular polysaccharide metabolic process	BP	3.07	0.05	0.27	13	44264
Potassium ion transport	BP	3.06	0.05	0.27	18	6813
Lateral plasma membrane	CC	3.06	0.05	0.27	8	16328
Defense response to Gram-positive bacterium	BP	3.06	0.05	0.27	8	50830
Positive regulation of exocytosis	BP	3.06	0.05	0.27	8	45921
Receptor regulator	MF	3.06	0.05	0.27	9	30545

activity						
Cardiocyte differentiation	BP	3.06	0.05	0.27	9	35051
Forelimb morphogenesis	BP	3.06	0.05	0.27	9	35136
Positive regulation of axonogenesis	BP	3.06	0.05	0.27	9	50772
Phagocytosis	BP	3.06	0.05	0.27	9	6909
Cell-cell adhesion	BP	3.06	0.05	0.27	54	16337
Transcription regulatory region sequence-specific DNA binding	MF	3.05	0.05	0.27	28	976
Regulation of macromolecule biosynthetic process	BP	3.05	0.05	0.27	434	10556
Chemical homeostasis	BP	3.04	0.05	0.27	107	48878
Oxidation-reduction process	BP	3.04	0.05	0.27	73	55114
Leydig cell differentiation	BP	3.04	0.05	0.27	4	33327
Trabecula	BP	3.04	0.05	0.27	4	61383

morphogenesis						
Positive regulation of interferon-gamma biosynthetic process	BP	3.04	0.05	0.27	4	45078
Positive regulation of insulin-like growth factor receptor signaling pathway	BP	3.04	0.05	0.27	4	43568
Mitogen-activated protein kinase kinase binding	MF	3.04	0.05	0.27	4	31434
Regulation of non-canonical Wnt receptor signaling pathway	BP	3.04	0.05	0.27	4	2000050
Chronic inflammatory response	BP	3.04	0.05	0.27	4	2544
Regulation of SMAD protein import into nucleus	BP	3.04	0.05	0.27	4	60390
Regulation of steroid hormone biosynthetic process	BP	3.04	0.05	0.27	4	90030

Histone H2A acetylation	BP	3.04	0.05	0.27	4	43968
Negative regulation of JUN kinase activity	BP	3.04	0.05	0.27	4	43508
Fibrillar collagen	CC	3.04	0.05	0.27	4	5583
Cellular response to chemical stimulus	BP	3.03	0.05	0.27	214	70887
Regulation of phospholipase activity	BP	3.03	0.05	0.27	16	10517
Positive regulation of locomotion	BP	3.02	0.05	0.27	39	40017
Fatty acid biosynthetic process	BP	3.02	0.05	0.27	20	6633
Telomere maintenance	BP	3.02	0.05	0.27	12	723
Steroid metabolic process	BP	3.01	0.05	0.27	38	8202
Golgi cisterna membrane	CC	3.01	0.05	0.27	14	32580
Peptide receptor activity	MF	3.01	0.05	0.27	21	1653
Epidermal growth factor receptor signaling	BP	3.00	0.05	0.27	31	7173

pathway						
Regulation of cytokine biosynthetic process	BP	3.00	0.05	0.27	17	42035
Positive regulation of MAPK cascade	BP	3.00	0.05	0.27	30	43410
Cellular chemical homeostasis	BP	3.00	0.05	0.27	80	55082
Nervous system development	BP	2.99	0.05	0.27	44	7399
Endopeptidase inhibitor activity	MF	2.99	0.05	0.28	28	4866
Endoplasmic reticulum-Golgi intermediate compartment	CC	2.98	0.05	0.28	11	5793
Extrinsic to internal side of plasma membrane	CC	2.98	0.05	0.28	11	31234
Regulation of striated muscle cell differentiation	BP	2.97	0.05	0.28	15	51153
Regulation of interferon-gamma production	BP	2.96	0.05	0.28	13	32649

Positive regulation of interleukin-1 beta production	BP	2.96	0.05	0.28	6	32731
Calcium activated cation channel activity	MF	2.96	0.05	0.28	6	5227
Regulation of oligodendrocyte differentiation	BP	2.96	0.05	0.28	6	48713
Neural tube development	BP	2.96	0.05	0.28	6	21915
Phosphatidylinositol 3-kinase binding	MF	2.96	0.05	0.28	6	43548
Middle ear morphogenesis	BP	2.96	0.05	0.28	6	42474
Positive regulation of cellular component biogenesis	BP	2.96	0.05	0.28	6	44089
Cellular homeostasis	BP	2.95	0.05	0.28	91	19725
ERBB signaling pathway	BP	2.94	0.05	0.28	31	38127
Cellular macromolecule	BP	2.94	0.05	0.29	36	70727

localization						
Regulation of ERK1 and ERK2 cascade	BP	2.93	0.05	0.29	21	70372
Regulation of hydrolase activity	BP	2.93	0.05	0.29	110	51336
Regulation of intracellular protein kinase cascade	BP	2.93	0.05	0.29	101	10627
Protein binding, bridging	MF	2.93	0.05	0.29	22	30674
Response to oxygen-containing compound	BP	2.92	0.05	0.29	135	1901700
Ventricular cardiac muscle tissue morphogenesis	BP	2.92	0.05	0.29	9	55010
Neuron-neuron synaptic transmission	BP	2.92	0.05	0.29	9	7270
Hindlimb morphogenesis	BP	2.92	0.05	0.29	9	35137
Regulation of cellular response to growth	BP	2.92	0.05	0.29	26	90287

factor stimulus						
Homophilic cell adhesion	BP	2.92	0.05	0.29	24	7156
Cellular response to organic substance	BP	2.92	0.05	0.29	184	71310
Protein tyrosine kinase activity	MF	2.92	0.05	0.29	25	4713
Locomotory behavior	BP	2.92	0.05	0.29	25	7626
Receptor activator activity	MF	2.91	0.05	0.29	7	30546
Negative regulation of gliogenesis	BP	2.91	0.05	0.29	7	14014
Golgi-associated vesicle	CC	2.91	0.05	0.29	7	5798
Positive regulation of proteolysis	BP	2.91	0.05	0.29	14	45862
Extracellular ligand-gated ion channel activity	MF	2.91	0.05	0.29	14	5230
Telomere organization	BP	2.91	0.05	0.29	12	32200
Negative regulation of	BP	2.91	0.05	0.29	8	30514

BMP signaling pathway						
Frizzled binding	MF	2.91	0.05	0.29	8	5109
Delayed rectifier potassium channel activity	MF	2.91	0.05	0.29	8	5251

BP, Biological process; MF, molecular function; cellular component; #, number of; GO ID, gene ontology identification.

Chapter 8 General discussion

The overarching purpose of this thesis was to determine the impact of strenuous exercise training on leukocyte telomere, DNA methylation and corresponding gene expression changes measured in context with cardiovascular health and fitness in humans. Accordingly, two cross-sectional studies were conducted to establish the differences in leukocyte telomere dynamics between endurance athletes, who perform copious amounts of aerobic endurance exercise, and apparently healthy controls. The second focus of this thesis was on the exercise training-induced changes to DNA methylation. In order to assess this, two training studies in young men, using a relatively new and effective type of exercise that improves cardiovascular health and fitness – sprint interval training – was used to assess the DNA methylation changes in leukocytes and sperm.

The results from each manuscript presented in this thesis have been discussed in Chapters 3,4, 6 and 7. This general discussion will integrate the results from the previous Chapters and provide direction for future investigations.

Endurance exercise training preserves leukocyte telomeres

The first study was designed to establish the long-term impact strenuous endurance exercise training has on leukocyte telomere length and whether any associations were mediated by cardiovascular risk factors and circulating markers of inflammation – interleukin-6 (IL-6), c-reactive protein (CRP), leptin, intercellular adhesion molecule-1 (ICAM-1) and e-selectin (E-selectin) (Chapter 3).[202] It was found ultra-marathon runners had significantly longer leukocyte telomeres compared to controls, but the difference was not explained by their excellent cardiovascular health.[202] The second study attempted to replicate the findings in independent cohorts of endurance athletes and healthy controls. In accordance with the previous results, the athletes had longer leukocyte telomeres despite being chronologically

five years older. Furthermore, resting heart rate and VO_{2max} emerged as important mediators of the longer leukocyte telomeres and up-regulated *TERT* and *TPP1* mRNA expression found in athletes. Werner *et al*. [200] revealed endurance athletes have up-regulated telomerase activity, which supports my data at the transcriptional level. Together, the data included in this thesis and data from other laboratories [200] suggests endurance exercise training may preserve leukocyte telomeres by up-regulating *TPP1* and *TERT* mRNA expression, which in turn, increases the processivity and activity of the enzyme telomerase, through the modulation of currently unknown molecular signalling cascades.

Inflammatory and oxidative stress pathways are prime candidate pathways that could play a role in exercise-induced telomere dynamics. Low-grade chronic inflammation and elevated reactive oxygen species (ROS) are phenotypes shared by many of age-related cardio-metabolic diseases associated with excessive telomere shortening.[312,620,621] Indeed, *in vitro*[168] and *in vivo*[622] experiments suggest inflammation shortens human fibroblast telomeres and culminates in cellular senescence through excessive ROS.[622] Interestingly, chronic exercise training lowers circulating markers of inflammation[36,280] and prevents excessive ROS by enhancing antioxidant capacity.[623,624] This considered and the fact that no statistically significant associations between telomere length and circulating markers of inflammation were observed in my first study, the endurance exercise training may attenuate telomere attrition by mitigating circulating ROS-induced telomere shortening and senescence, though this will require future investigations.

Exercise training reconfigures the DNA methylome

Telomere length is linked to chromatin structure, regulated by epigenetic modifications such as DNA and histone methylation and histone acetylation.[45,46,101,625] Therefore, the second theme of this thesis included two investigations on the influence of sprint interval training

(SIT) on DNA methylation in leukocytes (somatic cells) and sperm (germ cells). Given the more profound change to DNA methylation is observed after high-intensity exercise in humans,[526] the aim of Chapter 6 was to analyse the genome-wide leukocyte DNA methylation changes after four weeks of sprint interval training;[248] an emerging form of exercise training that improves cardiorespiratory fitness and cardiovascular health parameters rapidly and with less of a time demand than traditional longer duration, moderate intensity exercise.[626]

The four week SIT intervention was associated with marked changes across the leukocyte methylome, particularly in cardiovascular and cancer pathways.[248] These changes occurred concurrently with decreased and increased low-density lipo-protein cholesterol and cardiorespiratory fitness in young men, respectively. This study was the first to comprehensively characterise the genome-wide DNA methylation changes associated with intense aerobic exercise training and contributed the accumulating evidence indicating exercise training as a lifestyle strategy that effectively regulates the DNA methylome of multiple tissue types, such as muscle,[522,605] fat[523] and immune cells.[248] Many of the DNA methylation changes found in my study were found in genes enriched for molecular pathways that are also altered after longer (6-month) moderate-intensity aerobic exercise interventions. For example, Charlotte Ling's group found a six month exercise programme in middle-aged subjects elicited skeletal muscle CpG methylation changes in genes involved in the MAPK, Wnt, Hedgehog, calcium signalling pathways, respectively, as well as melanogenesis, progesterone-mediate oocyte maturation – identical pathways modulated in leukocytes after four weeks of SIT.[522] Moreover, the MAPK signalling pathway, known for its role in metabolism and mitochondrial function, is particularly modified after exercise training as genes enriched for this pathway were differentially methylated in three different tissues from three separate studies.[248,522,627] Therefore, exercise training seems to systemically influence

DNA methylation landscapes throughout numerous somatic cells in the body, potentially via a ROS-mediated mechanism.[583,628]

Some epigenetic modifications, including DNA methylation, are somewhat heritable, particularly at metabolic and paternally imprinted genes[614,615] – areas that escape the global de-methylation event upon fertilisation. In order to gain insight into whether exercise training affects the cells involved in the fertilisation process (germ cells – male sperm and female oocyte), and those cells that transfer the necessary genetic material that contributes to the offspring's genome, the aim of Chapter 7 was to ascertain whether the human sperm methylome is vulnerable to changes caused by three months of SIT.[627] Interestingly, SIT-induced genome-wide DNA methylation changes in human sperm occurred at CpG sites in genes enriched for known pathways involved in exercise training adaptations, such as MAPK signalling, PI3K-Akt signalling and ErbB signalling. The CpG sites with increased methylation after SIT were in genes with roles in many human diseases; the top 5 were schizophrenia, Parkingson's disease, autism, cervical cancer and leukaemia. As not all CpG sites escape the DNA methylation erasure associated with fertilisation, a disease association analysis was conducted, using the bioinformatics tool, DAVID,[538] on paternally imprinted CpG sites that are exempt from this process. Two of the 16 genes with DNA methylation modifications after SIT were associated with metabolic diseases that are prevented and controlled by regular exercise training – type 2 diabetes mellitus, hypertension and atherosclerosis (*IGF2* and *INS-IGF2*). Taken together, these novel investigations have provided evidence indicating SIT is an effective type of strenuous aerobic exercise that improves cardiovascular health and fitness, and reconfigures the DNA methylome in leukocytes and sperm. While these findings are not expected to directly change physical activity guidelines or policy, the premise of exercise-induced transgenerational inheritance of epigenetic modifications and the corresponding health outcomes may.

Limitations

There are a number of limitations associated with this thesis. Particular limitations associated with each study have already been discussed in Chapters 3,4,6 and 7. This section reiterates the important limitations from my studies.

Whole-blood leukocyte telomere and DNA methylation were analysed in context with exercise training. Leukocytes are made up of numerous subsets with different roles in immunity and these have different telomere lengths[31,309] and DNA methylation landscapes.[629] Leukocyte subset numbers or whole-blood leukocytes numbers were not analysed in my participants, which could have influenced results from the telomere and epigenetic studies. However, to minimise the effect of leukocyte subset variations, preprandial blood samples were collected from subjects in a rested state, 24 to 48 hours after previous physical activity or exercise sessions, to avoid any subset shifts or leucocytosis immediately after a bout of exercise training.[630,631] Additionally, it is unlikely that a particular type of leukocyte contributes to the overall health and fitness adaptations to exercise training and as such, the leukocyte telomere length and DNA methylation analyses performed in whole-blood leukocytes seemed a sensible starting point.

Chapters 3 and 4 included cross-sectional comparisons between endurance athletes and healthy controls. Although the athletes possessed longer leukocyte telomeres and increased expression of telomere-regulating genes (*TERT* and *TPP1*), causation cannot be assumed. Therefore, it is possible that regular endurance exercise does not maintain telomere length, rather the endurance athletes are genetically configured to have longer telomeres and elevated expression of telomere-regulating genes. It is, however, likely that the regular involvement in endurance exercise training causes up-regulation of telomerase activity and maintains

telomere length. Other data from the Charchar laboratory have indicated increased *TERT* mRNA expression in leukocytes after a 30-min bout of running at 80% of VO_{2peak}.[230]

The exercise induced telomere length maintenance is expected to involve the regulation of a network of genes and molecular pathways. An additional limitation of Chapter 4 is that only the seven known telomere length-regulating genes were chosen *a priori* for analysis. However, this type of analysis was chosen due to the limited amount of RNA and funding. Furthermore, protein analysis was not performed, as protein was not extracted from leukocytes and Western blot analyses would not have been possible given the amount of samples.

A common limitation associated with exercise studies is the lack of control for the impacts of diet. Particular diets rich in processed foods or vitamins are associated with shorter and longer leukocyte telomeres, respectively. Diet is also associated with particular DNA methylation changes in leukocytes. Therefore, it is possible that diet may have affected the results presented in this thesis.

Genome-wide DNA methylation analyses were performed using microarray technology (450K array, Illumina). This technology is prone to false positives and also false negatives and should be validated with other techniques with higher resolution. The 450K results from Chapter 6 were not validated by the use of the EpiTYPER (Sequenom) due to the problematic genomic location of the CpGs of interest. It was not financial feasible to validate the DNA methylation changes in Chapter 7. Whole genome bisulfite sequencing (WGBS) or methylated DNA immunoprecipitation sequencing (MeDIP-seq) are superior to the 450K array such that they have higher resolution.[632] These technologies are, however, significantly more expensive and less throughput compared to the 450K array,[632] making the use of these impossible due the limited budget associated with the PhD.

Future directions

Molecular exercise physiology research is in its infancy. Our understanding of the impact of exercise training on telomere biology and epigenetic modifications is fairly rudimentary. Consequently, there is a breadth of future directions appropriate for these topics and I have emphasised the important ones below:

- Efforts should be made to determine the influence of habitual exercise on telomere length and underlying telomere regulating genes and proteins, longitudinally over multiple years. The inclusion of a control group who does little or no exercise will help support the premise that exercise training prevents telomere length shortening. I will follow-up my athletes and controls from Chapter 4 in an observational study aimed at assessing telomere dynamics across multiple years (3 to 5 years). Additionally, analysing telomere length changes in context with epigenetic modifications may provide insight into the underlying molecular signals driving the telomere length changes or maintenance.

- Data from our laboratory has shown the major protein component of telomerase, *TERT* mRNA, expression is increased in leukocytes after an acute bout of aerobic exercise.[230] An extension of this work would be to measure telomerase activity and assess whether the accumulated and repeated exposure to exercise training may maintain telomeres through up-regulated telomerase activity. Data collection for this project is planned for late 2015.

- Considering exercise training prevents, manages and regresses many symptoms of age-related chronic diseases related to short telomeres, the identification of whether

exercise training exerts these functions through telomere and DNA methylation regulation is warranted. This would improve our understanding of the underlying mechanism by which exercise training stabilises health and may lead to novel pharmaceutical developments. Conducting randomised controlled trials with clinical populations, using different exercise intensities and new, robust analysis platforms could help achieve this aim.

- Functional studies analysing whether telomere length and/or DNA methylation (or other epigenetic modifications) changes directly influence gene expression and protein abundance are required. Ideally, these experiments would be performed *in vitro* and *in vivo*.

- Telomeres have a heritability estimate of approximately 0.62–0.86.[633] Therefore, it would be interesting to elucidate if, like DNA methylation changes, exercise training can affect telomere length in germ cells (female oocyte and male sperm).

- The most crucial question for the area of exercise epigenetics would be: are exercise-induced epigenetic modifications transgenerationally inherited by offspring and do these changes govern health and fitness? There are examples where particular *in utero* environmental stressors – plastics and pesticides (reviewed in[66]), undernourishment[634] and low protein diet[59,635] – modifies the rodent offsprings' epigenetic profile and causes metabolic consequences. Although rodent studies could help our understanding of the capacity for exercise to elicit such an affect, large human studies, across four generations would be ideal and would also have an exorbitant cost and effort related to them. The Framingham Heart Study is, however, a large, ongoing

study analysing cardiovascular disease risk factors in its third generation of human subjects and may soon provide insight on the transgenerational inheritance of heart disease.

Conclusions

This thesis has presented novel data from four investigations involving the analysis of telomere and DNA methylation in context with strenuous aerobic exercise and corresponding cardiovascular health and fitness adaptations. It was found that regular engagement in aerobic exercise training is associated with longer leukocyte telomeres with paralleled increased expression of leukocyte *TERT* and *TPP1* mRNA expression – two genes known for their role in telomere length maintenance. These anti-ageing effects were related to resting heart rate and VO_{2peak}. Moreover, short- and long-term SIT significantly modulates the leukocyte and sperm methylomes, respectively, in numerous genes and common signalling pathways modified in other somatic cells by other moderate-intensity exercise progammes.[522,523] The regulation of microRNAs by DNA methylation caused by SIT was also presented. Notably, these epigenetic changes occurred concurrently with improvements to cardiovascular health, fitness and performance. It is possible that telomere length maintenance and epigenetic modifications underpin the positive cardiovascular health and fitness benefits and prevention of premature ageing conferred by regular engagement in aerobic exercise. Further, exercise-induced germ line epigenetic modifications may influence the health of future generations and this paradigm deserves detailed attention in future investigations. These results may pave the way for more robust exercise training guidelines and national policies and contribute to the development of precision exercise.

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Appendices

A Online supplement to Exercise – putting action into our epigenome – published in *Sports Medicine*, 2014

Online Supplement to

Exercise – putting action into our epigenome

Short title: Exercise and epigenetics

Joshua Denham, Francine Z. Marques, Brendan J. O'Brien, Fadi J. Charchar

School of Health Sciences, University of Ballarat, Ballarat, VIC, Australia

Corresponding Author

Fadi Charchar, Room 228, F Building, Oppy Drive, University of Ballarat, Mt Helen, 3350.

P: (03) 5327 6098, Fax: (03) 5327 9602

Email: f.charchar@ballarat.edu.a

Electronic Supplementary Material

Table S1. Summary of relevant literature on exercise and miRNAs**.**

miRNA/s impacted				Cell type	Participant or animal phenotypes[a,b,c,d,e]	Exercise[f,g,h,I,j]	Reference
↑ or ↓	Acute effects	Chronic effects	Acute and chronic effects				
Human studies							
↑	•miR-125a •miR-210 •miR-222 •miR-29a (M only) •let-7d (F only)			Serum	50 (low VO_{2max}) and 50 (high VO_{2max}), 40-45, M+F, U, Low - ~102 ($ml \cdot kg^{0.75} \cdot min^{-1}$) high - ~148 ($ml \cdot kg^{0.75} \cdot min^{-1}$)	N/A	Bye et al.[513]

	•miR-21						
↓	•miR-151 (M only) •miR-652						
↑ ↓	Drosha Exportin-5 Dicer miR-133a miR-133b miR-181a miR-9 miR-23a miR-23b	miR-29b	miR-1 miR-31	Myocytes	9, 23, M, <2 hours per week, 44.1	Acute and chronic, aerobic (cycling), Acute – 60 (at ~70% of VO_{2max}); Chronic (4 days of 45 mins at ~75% of VO_{2max}, 2 days of 90 min at ~75% of VO_{2max} and 4 days of 6x5 min at ~95% of VO_{2max})	Russell et al. [425]
↑	miR-21-5p miR-24-2-5p			Leukocytes	8, 21.7, M, athletic (National level ski athletes), 74.8	Acute, aerobic (30 min treadmill run at 80%	Tonevitsky et al. [518]

	miR-27a-5p miR-181a-5p				VO_{2peak})	
↓		miR-486	Serum	8, 21.5, M, SED, 41.5	Acute and chronic, aerobic (cycling), acute – 60 min cycle at 70% of VO_{2max} and chronic – 30 min at 70% of VO_{2max}, 3, 4	Aoi et al. [514]
↑	miR-363 miR-338-3p miR-590-5p		Blood natural killer cells	13, 24, M, U, 48.2	Acute, aerobic (cycling), 10 x 2 min efforts at ~75% of VO_{2max} with 1	Radom-Aizik et al. [473]

	miR-7	min rest between efforts)
	miR-30e	
	miR-142-3p	
	miR-29c	
	miR-29b	
	miR-29a	
	miR-192	
	miR-142-5p	
↓	miR-126	
	miR-126*	
	let-7e	
	miR-130a	

	miR-151-5p miR-199a-5p miR-199a-3p miR-221 miR-223 miR-326 miR-328 miR-652				
↑	miR-126 (cycle VO_{2max} test, 4 hour cycle at 70% of VO_{2max}, Marathon race only)	Plasma	59, middle-aged, M+F, U, 23.6-59.8‖	Acute, aerobic (four groups: cycle VO_{2max} test, 4 hour cycle at 70% of VO_{2max}, marathon race	Uhlemann et al. [516]

	miR-133 (resistance training and marathon race only)					and resistance training)	
↑		miR-20a (acute after training)	miR-146a (acute after training) miR-222 (acute after training) miR-21 miR-221	Plasma	10, 19.1, M, athletic (rowers), 52	Acute (before and after training) and chronic, aerobic (endurance rowing), acute (cycle VO_{2max} test) and chronic – 60-180 (20-24 strokes per minute), 90 (in total)	Baggish et al. [474]
↑	miR-26b miR-1225-5p miR-338-3p miR-181c miR-363 miR-181a			Blood mononuclear cells	12, 22, M, U, 42.7	Acute, aerobic (cycling), 10 x 2 min efforts at ~75% of VO_{2max} with 1 min rest between efforts)	Radom-Aizik et al. [472]

	miR-181b
	miR-181a-2*
	miR-132
	miR-15a
	miR-939
	miR-7
	miR-140-5p
	miR-21*
	miR-940
↓	miR-652, let-7e
	miR-99b, miR-125b
	miR-125a-5p
	miR-151-3p
	miR-130a
	miR-126

	miR-199b-3p miR-23b miR-221 miR-199a-5p miR-584 miR-145 miR-31 miR-486-5p miR-151-5p miR-320 miR-451				
↓	miR-133a (after bed rest) miR-1 (after bed rest) miR-23a (acute exercise after bed rest)	Myocytes	12, 26.2, M, U, ~49 (after bed rest)	Chronic (7 days of bed rest – physical inactivity) and acute (after 7 days of bed rest), aerobic (knee extensor exercise), 45min	Ringholm et al. [433]

↑ ↓	••miR-451 ••miR-378 ••miR-26a ••miR-29a	Myocytes	56, 18-30, M	Chronic, resistance exercise, U, 5, 12	Davidsen et al. [493]
↑	miR-125a miR-183 miR-189 miR-432* miR-616 miR-637 miR-575	Myocytes	8, 23, M, SED, 47	Chronic, aerobic, 45 (at 70% of VO_{2max}), 4, 6	Keller et al. [417]

↓	miR-26b				
	miR-144				
	miR-101				
	miR-455				
	miR-338				
	miR-29b				
	miR-28				
	miR-1				
	miR-589				
	miR-451				
	miR-98				
	miR-92				
	miR15b				
	miR-133				
↓	miR-1	Myocytes	27, ~80, M+F	Chronic, resistance exercise, U, 2, 12	Mueller et al. [494]

↑	miR-520d-3p miR-1225-5p miR-1238 miR-125a-5p miR-145 miR-181b miR-193a-3p miR-197 miR-212 miR-223 miR-340* miR-365 miR-485-3p miR-505 miR-629*	Neutrophils	11, 22.2, M, U, 42	Acute, aerobic (cycling), 10 x 2 min efforts at ~75% of VO_{2max} with 1 min rest between efforts)	Radom-Aizik et al. [517]

	miR-638
	miR-939
	miR-940
↓	miR-20a miR-660
	miR-106a miR-96
	miR-20b
	miR-17
	miR-93
	miR-130b
	miR-16
	let-7i
	miR-107
	miR-126
	miR-130a
	miR-151-5p
	miR-185

	miR-18a miR-18b miR-194 miR-22 miR-363					
↑ ↓	miR-1 miR-133a	miR-1 miR-133a miR-133b miR-206	Myocytes	10, 30.5, RA, ~53	Acute and chronic, aerobic (cycling), acute – 60 min cycle and chronic – 60 to 150, 5, 12	Nielsen et al. [475]
↑	pri-miR-206 (Y+O) pri-miR-133a-2 (Y+O)		Myocytes	12 – 6 young (Y) and 6 old (O), young – 29 and old – 70	Acute, resistance exercise, 8 x 10 of knee extension exercise @	Drummond et al. [424]

↓	miR-1 (Y) pri-miR-1-2 (Y) pri-miR-133a-1 (Y)			70% of 1RM.	

Rodent studies

↑	miR-21 miR-144 miR-145	Cardiac myocytes	Wistar rats, U, F	Chronic, aerobic (swimming), 60, 5, 8	Ma et al. [484]
↓	miR-124				
↑	miR-126	Cardiac myocytes	Wistar rats, U, F	Chronic, aerobic (swimming), 60, 5, 10	Da Silva Jr et al. [489]

↑	miR-124	Brain	Wistar rats, U, M	Chronic, aerobic (treadmill running ~40% (low intensity group) or ~75 (high intensity group) of VO_{2max}), 30, 7, 2	Mojtahedi et al. [479]
↑	miR-21	Neuronal cells (spinal cord)	Sprague-Dawley rats, U, F	Chronic, aerobic (passive cycling), 60 (@45rpm), 5, 10‡	Liu et al. [478]
↑	miR-126 (SHR)	Myocytes	WKY and SHR, 12-week-old, U	Chronic, aerobic (swimming), 60, 5, 10	Fernandes et al. [486]
↓	miR-16 (SHR+WKY)				

	miR-21(SHR)				
↓	miR-494	Myocytes	C57BL/6J mice, 9-week-old, M	Chronic, aerobic (swimming), 7x15 min bouts with 5 min rests, 7	Yamamoto et al. [512]
↑	miR-27a miR-27b	Cardiac myocytes	Wistar rats, U, F	Chronic, aerobic (swimming), 60, 5, 10	Fernandes et al. [483]
↓	miR-143				
↑	miR-29a miR-29c	Cardiac myocytes	Wistar rats, U, F	Chronic, aerobic (swimming), 60, 5, 10	Soci et al. [482]
↓	miR-1 miR-133a miR-133b				

↑ ↓	miR-21 miR-15b	Neuronal cells (spinal cord)	Sprague-Dawley rats, U, F	Chronic, aerobic (passive cycling), 60 (@45rpm), 5, 10-31 day	Liu et al. [636]
↑ ↓	<u>Exercised mice</u> miR-21 <u>Immobilised mice</u> miR-680 miR-696 miR-705 miR-762 miR-696 miR-709	Myocytes	C57BL/6 mice, 8-week-old, U	Chronic, aerobic (treadmill running), 20-60, 5, 4	Aoi et al. [505]

	miR-720				
↑	miR-7* MM_264 miR-189 miR-377 miR-98 miR-126* miR-148b miR-338 miR-183 miR-489	Myocytes	Sprague-Dawley rats, 6-months-old, M	Chronic, Hind-limb suspension (immobilisation) for 2-7 days	McCarthy et al. [504]
↓	miR-23b miR-27b miR-333 miR-20a				

		miR-221 miR-222 miR-499 miR-505				
↑	miR-1 miR-107 miR-181		Myocytes	C57BL/6 mice, U, M	Acute, aerobic (treadmill running), 90	Safdar et al. [501]
↓	miR-23					
↑		pri-miR-1-2 pri-miR-133a-2 pri-miR-206	Myocytes	C57BL/6 mice, 10-weeks-old, M	Mechanical functional overload	McCarthy et al. [495]
↓		miR-1 miR-133a				

[a] = Number of subjects or breed of rodent.

[b] = Age of subjects (years).

[c] = Sex of subjects – Male (M) /female (F) or both genders (M+F)

[d] = Physical activity level – sedentary (SED), recreationally active (RA), athletic, unspecified (U)

[e] = maximal aerobic fitness (VO_{2max}) measured in $ml \cdot kg \cdot min^{-1}$.

[f] = Type of exercise intervention – acute, chronic or acute and chronic exercise or not applicable (N/A).

[h] = Minutes of exercise training per session.

[i] = Day/s or sessions of exercise per week.

[j] = Weeks (or months□) of exercise training (if any).

‡ = Indicates data from individuals from which the main findings originate from.

‖ = Indicates range of average VO_{2max} values from each of the participants in the cycle VO_{2max} test, four hour cycle at 70% of VO_{2max}, marathon race and resistance training group.

• = Participants with a low VO_{2max} compared to participants with a low VO_{2max}.

•• = Low responders compared to high responders.

Min = minutes; miR = micro-RNA; ↓ = decrease (s/d); ↑ = increase (s/d); * = passenger miRNA; pri = primary miRNA; VO_{2peak} = peak volume of oxygen; WKY = Wistar Kyoto rats; SHR = Spontaneously hypertensive rat.

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LEUKOCYTE TELOMERE LENGTH VARIATION DUE TO DNA EXTRACTION METHOD

Joshua Denham, Francine Z. Marques and Fadi J. Charchar*

Federation University Australia, Ballarat, Australia

Short title: Telomere length and DNA extraction

Emails: j.denham@federation.edu.au, f.marques@federation.edu.au, f.charchar@federation.edu.au

***Corresponding author**: Prof Fadi Charchar

Email: f.charchar@federation.edu.au

Ph: +613 5327 6098

Fax: +613 5327 9602.

Abstract

Background

Telomere length is indicative of biological age. Shorter telomeres have been associated with several disease and health states. There are inconsistencies throughout the literature amongst relative telomere length measured by quantitative PCR (qPCR) and different extraction methods or kits used. We quantified whole-blood leukocyte telomere length using the telomere to single copy gene (T/S) ratio by qPCR in 20 young (18-25yrs) men after extracting DNA using three common extraction methods: Lahiri and Nurnberger (high salt) method, PureLink Genomic DNA Mini kit (Life Technologies) and QiaAmp DNA Mini kit (Qiagen). Telomere lengths differences of DNA extracted from the three extraction methods was assessed by one-way analysis of variance (ANOVA).

Results

DNA purity differed between extraction methods used ($P = 0.01$). Telomere length was impacted by the DNA extraction method used ($P = 0.01$). Telomeres extracted using the Lahiri and Nurnberger method (mean T/S ratio: 2.43, range: 1.57 – 3.02) and PureLink Genomic DNA Mini Kit (mean T/S ratio: 2.57, range: 2.24 – 2.80) did not differ ($P = 0.13$). Likewise, QiaAmp and Purelink-extracted telomeres were not statistically different ($P = 0.14$). The Lahiri-extracted telomeres, however, were significantly shorter than those extracted using the QiaAmp DNA Mini Kit (mean T/S ratio: 2.71, range: 2.32 – 3.02; $P = 0.003$). DNA purity were associated with telomere length.

Conclusion

There are discrepancies between the length of leukocyte telomeres extracted from the same individuals according to the DNA extraction method used. DNA purity could be responsible for the discrepancy in telomere length but this will require validation studies. We recommend using the same DNA extraction kit when quantifying leukocyte telomere length by qPCR or when comparing different cohorts to avoid erroneous associations between telomere length and traits of interest.

Keywords: Telomeres, leukocyte, T/S ratio, qPCR, DNA extraction, High salt method

Background

In mammalian cells, telomeres are the repetitive sequence ($TTAGGG_n$) that prevent end-to-end fusion and maintains chromosomal stability [67]. Leukocytes circulate through many organs and their telomere length correlate to that of other tissues, including fat, muscle, skin and synovial tissue in humans [24,637,638]. Without the reverse transcriptase enzyme, called telomerase, somatic cell telomeres shorten during mitosis [68]. For this reason and due to ease of accessibility, leukocyte telomere length has become an important biomarker for cellular and biological age. Leukocyte telomere length is shorter in individuals with atherosclerosis [70], type 2 diabetes [143] and cancer [639], and is inversely associated with age and mortality risk [640]. Conversely, lifestyle factors such as a healthy diet, vitamin intake and exercise are associated with longer leukocyte telomeres [185,202,641].

There are a number of inconsistencies throughout the literature related to telomere length. For example, leukocyte telomere length quantified by real-time quantitative polymerase chain reaction (qPCR) using the telomere to single copy gene ratio (T/S ratio), in which the *36B4* gene is commonly used, varies dramatically throughout the literature and

associations between telomere lengths and diseases are sometimes equivocal [642]. The discrepancies could potentially be partially explained by the quantification of telomere lengths after extraction by different DNA extraction methods. We hypothesized that the DNA extraction method used to harvest DNA from leukocytes would impact their telomere length (T/S ratio) quantified by qPCR. Recently, it was demonstrated in a cohort of cancer patients and healthy individuals that telomere length of buffy coat leukocytes was dependent on the DNA extraction method used [642]. Whether telomeres from whole-blood leukocytes are influenced by using other DNA extraction methods remained to be tested. Also, the mechanism by which telomere length varies between extraction methods is not yet understood. Here we compared whole-blood leukocyte telomere length of DNA extracted using three different extraction methods; including two extraction kits that have not been studied in context with telomere length previously.

Methods

Sample collection

A resting blood sample was collected from 20 young (18–25yr) men. Participants self-reported that they were free from any infections and chronic diseases, and were otherwise healthy. Blood was drawn from the antecubital vein into EDTA tubes (BD Vacutainer, Australia) and was immediately stored on ice. DNA was extracted using three individual DNA extraction methods within 12 hours of collection to prevent any *ex vivo* impacts on telomere integrity. DNA yield and purity was checked using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Australia) before being stored at

-20°C. All blood collections were performed in the morning.

All participants gave written informed consent and this study was approved by the Federation University Australia Human Research Ethics Committee.

DNA extraction

DNA was extracted from whole-blood using the PureLink Genomic DNA Mini Kit (Life Technologies), QiaAmp DNA Mini Kit (Qiagen) and the Lahiri and Nurnberger (high salt) method, a non-commercial, relatively cheap, toxic reagent-free protocol [643]. Reagents for the Lahiri and Nurnberger method were prepared according to guidelines described elsewhere [643]. All DNA extractions were performed according the manufacturers recommendations by one researcher only.

Telomere assays

Relative telomere length was quantified by qPCR using the T/S ratio [644]. This is a relative measure of telomere length that is strongly correlated to telomere restriction fragments quantified by Southern Blot [644].The telomere assays were run on the ViiA7 Real Time PCR System (Life Technologies, Australia). Briefly, reactions were run in 384-well plate format with duplicates for samples, an exogenous positive and negative controls. Reaction were comprised of the following constituents: SensiFAST SYBR Lo-ROX master mix (Bioline, Australia), 300nmol/l of telomere-specific forward and reverse primers, or 300nmol/l of the single copy gene (*36B4*) forward and 500nmol/l of the *36B4* reverse primers and 30ng of DNA to make a total reaction volume of 10µl. The telomere assay primer-sets have been described elsewhere [14]. Difference of less than one cycle threshold (Ct) between duplicates was required. The telomere assays were repeated on 80% of samples on a separate day to assess the reproducibility of the data.

Statistics

Kolmogorov-Smirnov and Shapiro-Wilk test were performed and demonstrated the T/S ratios were normally distributed. An ANOVA was performed to determine differences in T/S ratio and DNA purity between the extraction methods studied. Pearson's Correlations were used to assess linear associations between DNA purity and T/S ratios. Statistical analyses were performed using the IBM SPSS statistics software (version 19) and significance was set at $P < 0.05$.

Results

DNA extracted from each of the three extraction methods showed significantly different purities ($P = 0.01$, Table 1). Specifically, Lahiri and Nurnberger and Purelink-extracted DNA had lower 260/280 ratios compared with the QiaAmp-extracted DNA (mean ± SEM, 1.7 ± 0.08 vs 1.93 ± 0.01, $P = 0.003$ and 1.78 ± 0.03 vs 1.93 ± 0.01, $P = 0.048$, respectively). There were no statistically significant differences between DNA 260/230 ratios of DNA extracted using the three different extraction methods ($P > 0.05$, Table 1).

The inter-assay coefficient of variation between independent replicates for the T/S ratio was 9.7% and r-value was 0.82 ($P < 0.001$, Figure 1). The intra-assay coefficient of variation was 1.5% for the telomere primer-set and 0.75% for the 36B4 primer-set, respectively. Therefore, the data showed acceptable reproducibility.

There were significant differences in the average T/S ratio between DNA extracted using the three methods (n = 20, $P = 0.01$). The QiaAmp and Purelink-extracted telomeres were not statistically different ($P = 0.14$). Similarly, telomeres of DNA extracted using the Lahiri and Nurnberger method (T/S ratio: 2.43, range: 1.57 – 3.02) and PureLink Genomic DNA Mini Kit (T/S ratio: 2.57, range: 2.24 – 2.80) did not differ ($P = 0.13$), but Lahiri and Nurnberger extracted telomeres were significantly shorter than those extracted using the QiaAmp DNA Mini Kit (T/S ratio: 2.71, range: 2.32 – 3.02, $P = 0.003$) (Figure 2). There

were no statistically significant correlations between the T/S ratios of DNA extracted using the three extraction methods ($P > 0.05$, Figure 3).

Finally, there were modest but statistically significant correlations between DNA purity and telomere length (260/280 and T/S ratio: r = 0.31, $P = 0.015$; and 260/230 and T/S ratio: r = 0.33, $P = 0.01$, Figure 4).

Discussion

Here we demonstrated leukocyte telomere length quantified by qPCR differs depending on the DNA extraction kit used to isolate the DNA. Furthermore, to our knowledge, we are the first to report telomere length could vary depending on the purity of DNA used for qPCR. Telomere length of DNA extracted from leukocytes using the Lahiri and Nurnberger method were different compared to those extracted using the QiaAmp DNA Mini method. The QiaAmp and Purelink-extracted telomeres were not statistically different. Moreover, telomere length of DNA extracted from the same individual using the three methods were not correlated. Whereas DNA extracted from leukocytes using the Lahiri and Nurnberger method demonstrated the largest variation in telomere length and the shortest average telomere length, the PureLink Genomic DNA Mini method had the least variation in telomere length and the QiaAmp DNA Mini method produced the longest telomeres. There was no statistical significant difference in the length of telomeres extracted using the Lahiri and Nurnberger and PureLink Genomic DNA Mini methods. Additionally, there was no statistically significant difference between telomeres extracted using the QiaAmp and Purelink methods.

Our findings extend previous data on relative telomere length differences between the QiaAmp DNA Mini method and other DNA extraction methods, specifically, the Gentra PureGene Blood Kit and a phenol/chloroform DNA extraction methods (both from Qiagen) [642]. Consistent with a previous report [642], the two column-based extraction methods used in

our study, the Purelink and QiaAmp protocols, yielded the smallest range of telomere lengths; the Lahiri and Nurnberger method had the largest telomere length range. Conversely, our data show column-based extraction methods produce longer telomeres compared to a high salt protocol. Particularly relevant is the Lahiri and Nurnberger method, which is a cheap and commonly used method before kits started to be used. In addition to extending previous findings [642], our data demonstrate other DNA extraction methods produce different leukocyte telomere lengths in a relatively modest number of participants.

Our novel finding that DNA purity (260/280 and 260/230 ratios) is correlated to telomere length suggests chemical contamination during DNA extraction could influence the qPCR reaction and telomere length. Interestingly, the QiaAmp-extracted DNA had the longest telomeres and the highest 260/280 ratio and the Lahiri and Nurnberger-extracted DNA had the shortest telomeres and lowest 260/280 ratio. The Lahiri and Nurnberger method contains high concentrations of salt which could contaminate extracted DNA. Certainly, additional studies will be required to confirm that differences in telomere length from DNA extracted using different extraction methods is due to DNA purity. We would, however, recommend caution when quantifying and comparing telomeres from DNA with different purities.

The leukocyte telomere length discrepancies observed in the present study could also be a result of telomere damage during the extraction process. The different reagents used in the extraction methods may affect telomere integrity during extraction. For example, both column-based extraction kits (QiaAmp and Purelink DNA Mini) include proteinase K and digestion buffers which effectively lyse proteins and inactivates nucleases. The Lahiri and Nurnberger (high salt) method [643], however, uses non-toxic reagents and a non-ionic detergent, octylphenoxypolyethoxyethanol (Nonidet P-40, Signma-Aldrich), and unlike the

column-based kits, may cause residual reagents in isolated DNA during the extraction procedure. This would lead to a decreased DNA purity which might inevitably affect telomere length assessed by qPCR. Shearing of DNA and low molecular weight may also explain differences in telomere length amongst different extraction methods [642]. Both the QiaAmp and Purelink DNA Mini kits isolate DNA 20–50kb in length. Alternatively, telomere structure may influence telomere length measured by qPCR. Telomeres can form quadruplex structures [645] and telomeric DNA is vulnerable to oxidative damage [169], which may be influenced by some reagents or high concentrations of Na^+/K^+ [646,647]. This may inevitably contribute to altered telomere amplification during qPCR and consequently telomere length. Epigenetic modifications, such as histone acetylation and DNA methylation, are abundant at telomeres and sub-telomeric regions [45], and could also modify the DNA amplification during qPCR. It has been suggested that telomere length differences arising from different DNA extraction methods used are likely to be related to DNA quality and not qPCR amplification effects [642], yet this was not directly analysed. We, however, have demonstrated DNA purity correlates to leukocyte telomere length in healthy young men.

Our results have implications to already published and future research. Any investigations involving telomere length quantification should be required to extract DNA using the same method in order to make justified conclusion regarding the phenotypic association with telomere length. This may be problematic for meta-analyses and large studies that are analyzing a phenotype in context with telomere dynamics, as they involve large data-sets from multiple laboratories, yet the utilization of different extraction methods could yield misleading results. Telomere length has been identified as a clinical relevant prognostic biomarker of disease and disease risk [18,19,176,648], and according to our data and that of others [642] the use of different extraction methods across multiple time-points should be

avoided. Moreover, as DNA purity could influence telomere length, it could be advantageous to control for DNA purity in statistical analyses when comparing telomere length.

A limitation of our study is that we quantified leukocyte telomere length of 20 young men, a relatively modest number. We have, however, clearly shown that even in a small cohort of young men leukocyte telomere length is impacted by the DNA extraction method used.

Future work should aim at replicating our findings and previous published data [642] suggesting DNA extraction methods influence leukocyte telomere length quantified by qPCR, to confirm results and identify explanations for the discrepancies. The investigation into the effect of other commercial and non-commercial DNA kits on leukocyte and additional cell-type telomere lengths is also warranted. Establishing the cause of the different telomere lengths from different extraction kits is also required. Lastly, it would be beneficial to validate which DNA extraction method is the most valid estimate of telomere length quantified by other methods such as Southern Blot or quantitative fluorescent in situ hybridization (Q-FISH). Interestingly, QiaAmp-extracted leukocyte telomeres were, on average, shorter compared to telomeres extracted by both phenol/chloroform and PureGene protocols when quantified by qPCR but were similar to phenol/chloroform-extracted telomeres when quantified by Southern Blot [642].

Conclusions

By comparing the length of telomeres extracted from whole-blood leukocytes using three different extraction methods, we have demonstrated that the method of DNA extraction impacts leukocyte telomere length possibly by yielding different DNA purity. We recommend the adherence to a single type of DNA extraction method when performing comparative and prospective telomere length studies in context with health and disease traits.

List of abbreviations

qPCR - quantitative polymerase chain reaction

T/S ratio – telomere to single copy gene

LSD – least significant difference

Ct – cycle threshold

Competing interests

The authors have no competing financial or non-financial competing interests to declare.

Authors' contributions

JD carried-out participant recruitment, sample collection, all telomere assays, data analysis and the writing of the manuscript. FM contributed to the study design, writing and reviewing of the manuscript. FC supplied the materials for telomere assays, contributed to the study design, the writing and reviewing of the manuscript.

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Figure legends

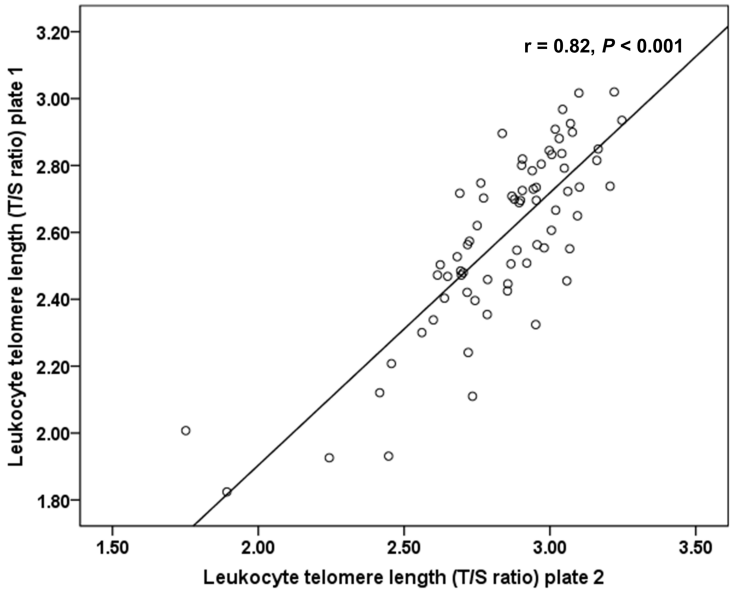


Figure 1. Linear correlation between telomere assays. Telomere assays were repeated on 80% of samples on a separate day to assess the reproducibility of the data. Telomere length (T/S ratio) data showed acceptable reproducibility (r = 0.82, $P < 0.001$).

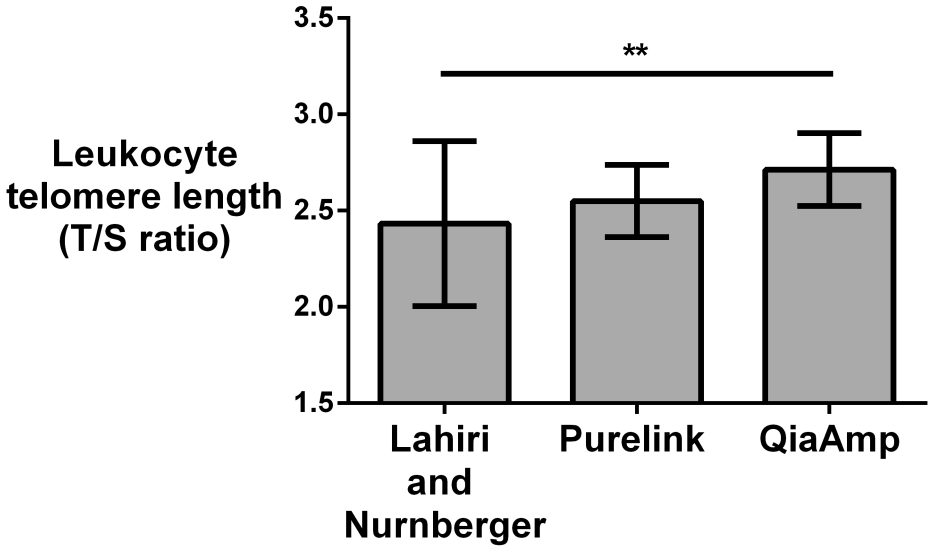
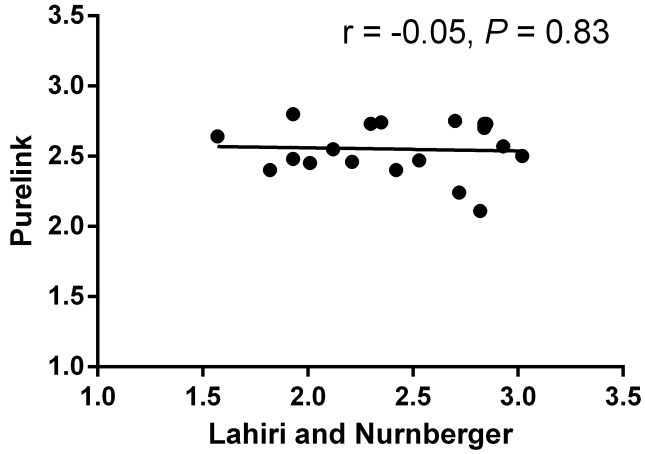
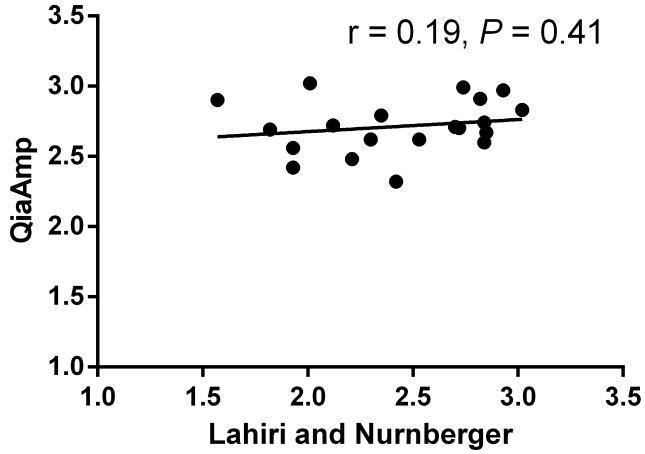


Figure 2. DNA extraction method impacts leukocyte telomere length (T/S ratio). Bars are mean telomere length and error bars are standard deviations.

DNA was extracted using the Purelink, QiaAmp and Lahiri and Nurnberger methods, respectively. $^{**}P = 0.003$.


r = -0.05, P = 0.83
Purelink
Lahiri and Nurnberger
3.5
3.0
2.5
2.0
1.5
1.0
1.0
1.5
2.0
2.5
3.0
3.5


r = 0.19, P = 0.41
QiaAmp
Lahiri and Nurnberger
3.5
3.0
2.5
2.0
1.5
1.0
1.0
1.5
2.0
2.5
3.0
3.5

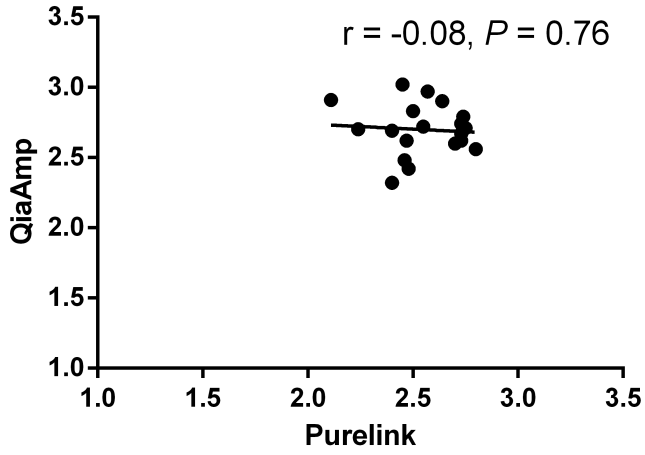
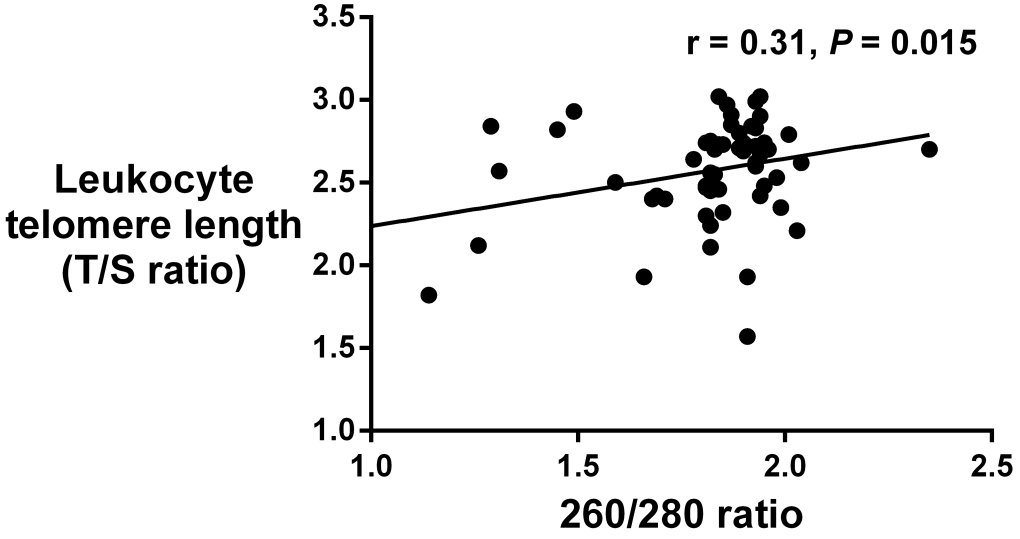

r = -0.08, P = 0.76
QiaAmp
Purelink
3.5
3.0
2.5
2.0
1.5
1.0
1.0
1.5
2.0
2.5
3.0
3.5

Figure 3. Correlations between 20 individuals telomeres from DNA extracted from three different extraction methods. The x and y axis represents leukocyte telomere length (T/S ratio).



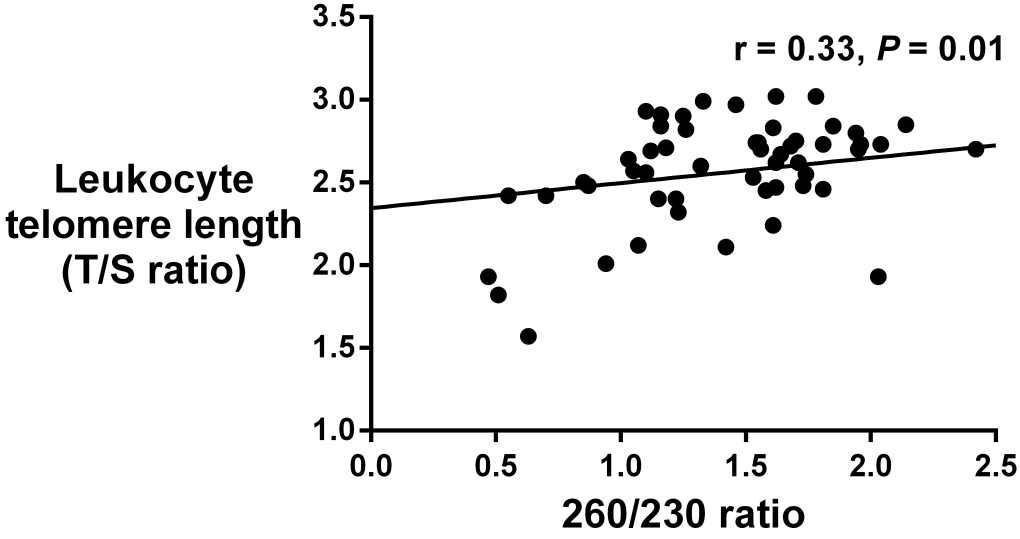


Figure 4. Correlation between leukocyte telomere length (T/S ratio) with DNA purity (260/280 and 260/230 ratios).**Table**

Table 1. DNA quality of samples extracted using three extraction methods.

Extraction method	***DNA quality***	
	260/280	**260/230**
Lahiri and Nunberger	1.7 ± 0.08†	1.45 ± 0.23
Purelink	1.78 ± 0.03‡	1.62 ± 0.1
QiaAmp	1.93 ± 0.01	1.41 ± 0.09

Data are expressed as mean ± SEM from 20 DNA samples extracted using three different extraction methods.

† Lahiri and Nunberger vs QiaAmp ($P = 0.003$)‡ Purelink vs QiaAmp ($P = 0.048$)

C Four weeks of sprint interval training improves 5 km run performance – published in the *Journal of Strength and Conditioning Research*, 2015.

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Four weeks of sprint interval training improves 5 km run performance

Short title: SIT improves 5 km run performance

Authors: Joshua Denham, Simon A. Feros and Brendan J. O'Brien

Faculty of Health Sciences, Federation University Australia, Mt Helen, 3350, Australia

Corresponding author: Joshua Denham, Faculty of Health Sciences, Federation University Australia, University Drive, Mt Helen, 3350. Email: j.denham@federation.edu.au, Ph: +61 3 5327 6660.

ABSTRACT

Sprint interval training (SIT) rapidly improves cardio-respiratory fitness but demands less training time than traditional endurance training. While the health and fitness gains caused by SIT are understood, the effect of short-term SIT on 5 km run performance remains elusive. Thirty healthy untrained participants (aged 18–25 years) were allocated to a control (n = 10) or a SIT (n = 20) group. SIT involved three to eight sprints at maximal intensity, three times a week for four weeks. Sprints were progressed to eight by the 12th session. All participants completed a 5 km time-trial on a public running track and an incremental treadmill test in an exercise physiology laboratory to determine 5 km run performance and maximum oxygen uptake, respectively, before and after the 4 week intervention. Relative to the controls, sprint interval trained participants improved 5 km run performance by 4.5% ($p < 0.001$) and this was accompanied by improvements in absolute and relative maximum oxygen uptake (4.9%, $p = 0.04$ and 4.5%, $p = 0.045$, respectively). Short-term SIT significantly improves 5 km run performance in untrained young men. We believe SIT is a time-efficient means of improving cardio-respiratory fitness and 5 km endurance performance.

KEYWORDS: Time-trial, training load, SIT, VO_{2max}

INTRODUCTION

Sprint interval training (SIT) is a form of run or bicycle training of short, maximal efforts of approximately 30 s, repeated several times interspersed with 3-5 mins of recovery, aimed at improving cardio-respiratory fitness and performance. SIT is proposed as an alternative mode of training to traditional constant-rate training. SIT improves cardio-respiratory fitness to a similar degree as constant-rate training but requires 90% less weekly energy expenditure and 66% less total training time [626].

The efficacy of SIT to improve run endurance performance, however, is only beginning to be elucidated. Ten weeks of two times a week SIT involving twelve 30 s efforts improved 3 km time-trial by 3.4% in moderately trained runners [649]. SIT may be more efficacious at improving endurance performance than both continuous running and other high-intensity interval training (HIT) programmes. For example, 20 individuals who completed seven to twelve efforts of 30 s sprints thrice weekly for 6 weeks, improved 3 km run performance greater than individuals who completed constant rate running or four to six 4 minute efforts at 3 km average running velocity over 6 weeks [609]. Others have, however, demonstrated recreationally active participants similarly improve 2 km run performance after either 6 weeks of SIT (4.6%) or constant rate running (5.9%) [608]. Therefore, SIT seems to enhance run performance over 2-3 km, but whether these endurance benefits improve run performance in untrained participants over longer distances is unknown. Furthermore, the lack of inclusion of experimental controls in previous studies [608,609,649] makes understanding the efficacy of SIT at improving run performance difficult.

Run performance is important for individuals of average cardio-respiratory fitness who aspire to improve their fitness and performance quickly and efficiently. The impact of SIT, in the form of running, on untrained subjects' 5 km run performance and maximum oxygen uptake ($\dot{V}O_{2max}$), has not been reported previously. Moreover, considering the importance of cardio-respiratory fitness in ball sports such as soccer, Australian Rules Football and cricket [650-652], where participants run distances often exceeding 5 km, there is immense coaching emphasis on reducing training load to minimise injury risk, but maximise training time to focus on other skills important to the sport. To that end, the impact of SIT on endurance performance exceeding distances previously investigated warrants attention. Furthermore, SIT training simultaneously develops endurance, speed and repeated sprint capability [609], all important fitness components of team-sports.

Therefore, the purpose of our study was to determine whether 4 weeks of three sessions a week SIT improves cardio-respiratory fitness and 5km run performance. We hypothesised that SIT would significantly improve $\dot{V}O_{2max}$ and 5km run performance.

METHODS

Experimental approach to the problem

This study is a controlled trial, where healthy participants performed either 4 weeks of SIT or no training, to evaluate its effect on 5 km run performance and $\dot{V}O_{2max}$. $\dot{V}O_{2max}$ was measured during a maximal treadmill test conducted in the university exercise physiology laboratory and the 5 km time-trial was assessed at a public running track. These tests were performed on

separate days within one week of commencing the initial SIT session and within one week after the final SIT session.

Subjects

Thirty, apparently healthy young men (18–25 years) were recruited for this study. Participants were initially screened to ensure they were not currently engaging in any structured high-intensity aerobic exercise training. All participants reported they had not completed any structured aerobic exercise training in the past year. 20 participants were allocated to the SIT (cases) group and 10 participants were allocated to the control group.

Participants gave written informed consent and this study was approved by the University's Human Research Ethics Committee.

Procedures

Participant $\dot{V}O_{2max}$ was assessed during a maximal treadmill test, by pulmonary analysis conducted in an exercise physiology laboratory at the university. Before the $\dot{V}O_{2max}$ test participants were fitted with a two-way breathing valve (Hans Rudolph) and expired air was collected into an online metabolic system (Moxus) for gas (O_2 and CO_2) analysis. The metabolic system was calibrated before each test using ambient air and gas of known composition. Participants were given a standardised 3 min warm-up at 10 $km \cdot h^{-1}$. The $\dot{V}O_{2max}$ test commenced at 10 $km \cdot h^{-1}$, and treadmill speed was progressively increased by 1 $km \cdot h^{-1}$ every second minute. The treadmill speed was progressively increased until volitional

exhaustion. $\dot{V}O_{2max}$ was determined as the highest O_2 value averaged over 60 s. _ENREF_7All laboratory testing was performed preprandially at the same time of day (8–10 AM). Participants were encouraged to stay hydrated the night before and morning of testing. Within a week of the $\dot{V}O_{2max}$ assessment each participant completed a supervised 5 km run time-trial on a flat running track in a local park. Briefly, participants completed a short 10 min warm-up including some light aerobic exercise and dynamic stretches. Participants were supervised and instructed to run maximally at their own pace.

Cases completed a standardised SIT programme performed three times a week over four weeks (total of 12 sessions). The sprint duration and recovery period was controlled at 30 s and 4 mins (passive), respectively. Participants were requested to run maximally for each 30 s sprint. An Accredited Exercise Physiologist (with Exercise and Sport Science Australia) provided participants with verbal encouragement and supervised each training session. SIT was progressively overloaded by increasing the number of sprints (Table 1). Before each training session, participants completed a standardised warm-up entailing a 5 min aerobic warm-up, dynamic stretches and some short (20 m) runs at approximately 70, 80, 90 and 100% effort.

<<Table 1 about here>>

Cases and controls were instructed not to deviate from their current physical activity and exercise habits during the 4 week intervention period. All participants were requested not to change their diet.

Statistical analyses

All statistical analyses were performed using SPSS (version 20). Data were tested for normality and non-parametric data was log-transformed before further analysis. T-tests were used to examine fitness and performance changes after SIT, relative to the control group. Significance was set at $p < 0.05$.

RESULTS

Test-retest reliability data is outlined in Table 2. The 5 km time-trial test had excellent reliability with an intraclass correlation coefficient of 0.99 and a 3.4% coefficient of variation.

<<Table 2 about here>>

Table 3 outlines the fitness and performance changes after 4 weeks of SIT. While absolute and relative $\dot{V}O_{2max}$ increased after SIT in cases (4.9%, $p = 0.04$ and 4.5%, $p = 0.045$, respectively), absolute and relative $\dot{V}O_{2max}$ were unchanged in controls (0.6%, $p = 0.73$ and -0.6%, $p = 0.72$, respectively). Relative to the control group who showed a marginal increase in 5 km time-trial performance (-2%, $p = 0.20$, Table 3), cases had a significant improvement in 5 km time-trial performance by an average of 65 s (-4.5%, $p < 0.001$) following 4 weeks of SIT (Table 3).

<<Table 3 about here>>

DISCUSSION

The purpose of this study was to investigate whether a 4 week SIT intervention improves 5 km run performance in previously untrained males. To our knowledge, we are the first to

demonstrate that 5 km run performance is significantly improved following short-term (4 weeks) SIT. Considering the marginal but not statistically significant improvement in the controls (31 s, 2%), the marked (96 s, 6.5%) improvement after 4 weeks of SIT translated to a mean 65 s (4.5%) faster 5 km run performance in cases. Notably, 5 km run performance was improved in conjunction with increased $\dot{V}O_{2max}$.

The increased 5 km run performance in our study is similar to previously observed improvements after traditional constant-rate endurance training. It was reported, an intervention of three running sessions per week at 75% of $\dot{V}O_{2max}$ for 6 weeks improved 5 km run time by approximately 80 s (~5%) in 39 untrained individuals [653]. Additionally, others have shown 5 km time-trial improved by 78 s (5%) in a group that ran for 20 mins three times per week for 6 weeks, initially starting at 0.8 $km \cdot hr^{-1}$ below their individual lactate threshold speed with progression to 0.8 $km \cdot hr^{-1}$ above their individual pre-training lactate threshold speed [654]. Importantly, a control group was not included in these studies to establish the effect of being familiar with the 5 km distance, which we established was 31 s. Collectively, it appears SIT improves 5 km run performance to a similar extent than constant-rate training and in a quicker time frame (4 vs. 6 weeks) and training duration (249 mins vs. 360 mins [654]).

Our data showing SIT enhances 5 km run performance corroborates others' showing SIT enhances 2 km [608] and 3 km [609,649] run performance in recreationally trained individuals. We are the first to demonstrate 4 weeks of three times per week SIT, in the form of running, significantly improves 5 km run performance – a prestigious and Olympic running distance. Interestingly, the impact of SIT on 5 km endurance performance is established in other exercise modalities. A similar short-term (2-week) cycle SIT intervention improved 5 km

cycle performance to a similar magnitude of our protocol (5.2%) [655]. Whether SIT improves run performance over longer distances warrants attention. Additionally, whether SIT benefits already well-trained runners is yet to be fully understood, as data has, to date, been equivocal [656,657]. Nevertheless, we verify SIT as an effective means of improving running endurance performance in untrained young men.

_ENREF_3

The improvement in run performance was accompanied by a corresponding improvement in $\dot{V}O_{2max}$. Although previous constant-rate training studies have demonstrated participants increased their $\dot{V}O_{2max}$ to a similar magnitude observed in the our study (3.8–7.7%), they also showed marked improvements in their participants lactate threshold, which could contributed to the improvements in run performance [653,654]. Alternatively, an improvement in cardiac output may have facilitated the improvement in 5 km run speed, as opposed to improvements in muscle oxidative capability, based on the concepts $\dot{V}O_{2max}$ is chiefly governed by the maximal cardiac output and the lactate threshold by muscle oxidative capability [658]. Other mechanisms by which SIT may enhance 5 km run performance include improved run economy, running mechanics and potassium regulation. It was previously reported that moderately-trained runners' run economy was improved by 7% after 4 weeks of SIT, but these subject did not improve their 10 km time-trial performance [657]. Moreover, increases in knee flexor endurance, coupled with decreases in knee flexion torque and knee flexion/extension ratios are also adaptations associated with sprint training that could have contributed to the improvement in 5 km run performance observed in our study [659]. Others have shown in well-trained runners, SIT enhanced key components of the Na^+/K^+ pump that would aid to minimise disturbances in nerve membrane potential and maintain sprint performance, and this was associated with an approximate 3% improvement in 3 km and 10

km run performance [656]. The increase in muscle oxidative and anaerobic enzyme activity are adaptations gained from SIT and is another possible mechanism for the facilitated endurance performance observed in participants from our study [543,660-662].

A limitation of our study is that both experimental and control groups did not complete any familiarisation sessions before 5 km time-trial testing. We did, however, assess the reliability of the 5 km time-trial test using the controls and found it had excellent reliability with values substantially less than the observed training changes, thereby excluding the possibility that the SIT-induced performance benefits were largely due to a learning effect. We did not include females in this study. Some of the benefits gained from SIT seem to be dependent of gender. For example, males had greater muscle protein synthesis of proteins important for mitochondrial biogenesis after nine sessions of SIT compared to their female counterparts [663]. Given these muscle adaptations are vital for endurance performance [661,662,664], whether short-term SIT improves 5 km run performance in females is left for future investigations.

Future research could focus on optimising training variables, such as the recovery time between sprints, number of sprint repetitions, time required of each sprint, number of sessions required per week and the optimal length of SIT. In fact the efficiency of HIT in evoking positive adaptations may even be more potent than originally envisaged. It was recently revealed one maximal 4 min effort at 90% of heart rate maximum on a cycle ergometer, performed three times a week for 10 weeks improves $\dot{V}O_{2max}$ by an average of 10% [665].

In conclusion our data reveals SIT is a highly effective strategy to improve endurance running quickly in previously untrained young men.

PRACTICAL APPLICATIONS

We believe our findings have practical implications for recreational runners and for team-sport coaches who aim to improve the endurance of their players efficiently within periodised programmes and limited time-frames associated with pre-season training. Moreover, SIT is feasible for and may appeal to individuals who perceive time as an obstacle to exercising, as it can rapidly enhance not only cardio-respiratory fitness but also endurance performance.

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TABLES

Table 1. Description of the 4-week SIT regimen.

Week	Session #	Training load (sprints)*	Training sprint time (s)	Total session time (mins)
1	1	3	90	9.5
	2	4	120	14
	3	5	150	18.5
2	4	4	120	14
	5	5	150	18.5
	6	6	180	23
3	7	5	150	18.5
	8	6	180	23
	9	7	210	27.5
4	10	6	180	23
	11	7	210	27.5
	12	8	240	32
	Total time	66	1980	249

All sprints were completed at maximum intensity and separated by 4 minutes of passive rest.

Legend: # number.

Table 2. Test-retest reliability data for the 5 km time-trial test.

Variable	**Mean trial 1**	**Mean trial 2**	**Mean difference**	**Mean difference (%)**	***P*-value (mean change)**	**ICC**	**CV (%)**
5 km time-trial (s)	1478.1 ± 349.9	1447.5 ± 347.2	-30.6 (-78.02–17.2)	-2.1	0.18	0.99 (0.95–0.99)	3.4 (2.4–6.4)

Results of the 5 km time-trial test in the control group (n=10), reliability as measured by mean difference, mean difference as a percentage of the mean of 2 trials, intraclass correlation (ICC), *p*-value (paired *t*-test), and typical error as a coefficient of variation (CV) percentage (%). Upper and lower confidence intervals are expressed in parentheses; confidence intervals are set at 95%. SDs are represented after the mean of trials 1 and 2.

D Low augmentation index in endurance athletes: a role for cardiorespiratory fitness – submitted for publication in the *Journal of Human Hypertension*

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Low augmentation index in endurance athletes: a role for cardiorespiratory fitness

Running title: Haemodynamics in endurance athletes

Joshua DENHAM[a], Nicholas J. BROWN[b], Maciej TOMASZEWSKI[c], Brendan J. O'BRIEN[b], Fadi J. CHARCHAR[a*]

[a]Faculty of Science and Technology, Federation University Australia, University Drive, Mt Helen, Victoria, Australia. [b]Faculty of Health, Federation University Australia, University Drive, Mt Helen, Victoria, Australia. [c]Department of Cardiovascular Science, University of Leicester, UK

[*]Corresponding author: email: f.charchar@federation.edu.au; phone: +613 5327 6098; fax: +613 5327 9242.

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ABSTRACT

Endurance exercise training is improves cardiovascular health and reduces mortality risk. Augmentation index [AIx] indicates the adverse loading exerted on the heart and large arteries and predicts future cardiovascular disease. The first purpose of this study was to determine whether central blood pressure (BP) and AIx differs between endurance athletes and recreationally active controls, and in the event of any differences, a second aim was to identify whether any potential differences can be attributed to cardiovascular risk factors, physical activity parameters or cardiorespiratory fitness.

Forty-four endurance athletes and 50 recreationally active controls completed physical activity/inactivity questionnaires, and underwent central BP and AIx measurements by non-invasive applanation tonometry. $\dot{V}O_{2peak}$ was assessed during graded exercise testing.

Relative to controls, athletes had significantly lower brachial and central BP (all $p \leq 0.01$). In age, height and heart rate-adjusted analyses, athletes had significantly lower AIx ($p = 0.001$), a difference ameliorated after adjustment BP parameters and $\dot{V}O_{2peak}$ ($p = 0.31$). Relative to men with low $\dot{V}O_{2peak}$, those with moderate and high $\dot{V}O_{2peak}$ had significantly lower AIx ($p = 0.007$). No statistically significant associations between fitness tertiles and AIx in women ($p = 0.2$).

In conclusion, the lower AIx in endurance athletes is partly mediated by $\dot{V}O_{2peak}$. Considering the comparable AIx between moderately and highly fit men, the strenuous, high-volume exercise training practiced by endurance athletes to maximise $\dot{V}O_{2peak}$ may not be required to prevent adverse loading on the heart and large arteries, rather exercise training facilitating an achievable $\dot{V}O_{2peak}$, above 45 $ml \cdot kg^{-1} \cdot min^{-1}$ is sufficient.

Key words: Central blood pressure, cardiovascular health, $\dot{V}O_{2peak}$

INTRODUCTION

High central (aortic) blood pressure (BP) is related to cardiovascular disease and is predictive of future adverse cardiovascular events – more so than brachial BP.[666-668] Arterial stiffening occurs with ageing and contributes to an increased load inflicted on the central arterial and left ventricular walls, and hence an increase in risk of organ damage, coronary artery disease and heart failure.[669] Aortic augmented pressure (AP) and augmentation index (AIx) are surrogate measures of the load inflicted on the central arterial and left ventricular walls,[670] such that AP is the difference between the first and second systolic peaks and AIx is AP expressed as a percentage of pulse pressure. Indeed, AIx is an independent marker of premature coronary artery disease[671] and AIx is a strong, independent predictor of congestive heart failure[672] and all-cause mortality[673] in healthy and clinical populations.

Endurance athletes have favourable cardiovascular risk factors, including low BMI, resting heart rate, cholesterol and serum C-reactive protein.[202,279,280,674,675] Although long-term endurance exercise training typically attenuates these cardiovascular risk factors, recent findings indicate chronic, strenuous endurance exercise may cause stiffening of the heart and large arteries.[675-677] The potential for long-term extensive endurance exercise to stiffen the heart and central arteries could be explained by an increased loading to the these structures; owing to elevated central BP and/or AIx.

The effect long-term and regular endurance exercise training has on central BP and AIx is currently unclear, due to contrasting findings between studies.[674,678-682] For example, some revealed endurance athletes have lower[678] or comparable[674,679,682] AIx when compared to healthy controls. Consistent with the AIx, data on central blood pressures between athletes and controls are equally equivocal.[674,678,680] The lack of adjustment for confounding factors

(age, height, resting hear rate or brachial BP) or differences in subject ages, diets, cardiorespiratory fitness levels, or study methodologies (e.g. pre-race haemodynamic measurements), including statistical power, could contribute to the discordant findings in previous literature. Consequently, more research is needed to determine whether endurance athletes have altered central BP and AIx. Identification of the cardio-vascular risk factors of which endurance exercise potentially mediates AIx would shed light on the mechanism by which exercise may influence adverse excessive loading of the heart and large arteries.

Therefore, the primary aim of our study was to investigate whether central BP and AIx are different between endurance athletes and healthy controls. Upon identification of any differences, a secondary aim was to identify which cardiovascular risk factors or physical activity/fitness parameters mediate the effect on central BP and AIx in endurance athletes and controls. We hypothesised endurance athletes possess lower central BP and AIx compared to their less-active peers and that particular physical activity parameters or $\dot{V}O_{2peak}$ would mediate any differences.

MATERIALS AND METHODS

Participants

All subjects were recruited from the general public and University by flyers, Internet advertising and word-of-mouth. Subjects were non-smokers, free from age-related diseases (including cardiovascular disease, type 2 diabetes and cancer) and not taking any medications. Forty-four male and female endurance-trained athletes and recreationally active controls aged 18–55 y were recruited for cross-sectional analysis. Endurance-trained individuals (athletes) had trained >3 days a week for a minimum of one year and consisted of four cyclists, 24 triathletes, 14 runners and two ultra-marathon runners; including 21 recreationally competitive, 12 State, three National and eight International level athletes. The

healthy controls were recreationally active but were not engaging in any structured resistance or aerobic exercise training. Athlete and control characteristics are outlined in Table 1.

All participants gave written informed consent and this study was approved by Federation University Australia's Human Research Ethics Committee.

Procedures

Athletes and controls attended one testing session conducted in the morning (8–10AM) after an overnight (10 hour) fast. Subjects were requested to abstain from caffeine, alcohol and physical exercise 24 hours before testing. Physical activity readiness questionnaire (PARQ), the self-administered International Physical Activity Questionnaire (IPAQ) Long form,[299] Perceived Stress Scale (PSS)[300] and a medical questionnaires were completed. Data cleaning and analysis was performed according to the IPAQ guidelines and average weekly Metabolic Equivalent of task (MET) – minutes and sitting were calculated and included as continuous variables in statistical analyses. Height, weight and body mass index (BMI) were recorded and subjects were seated for approximately 10 minutes before BP assessment. Brachial and central BP was averaged from three separate measurements, taken one minute apart with subjects seated. The same researcher (JD) recorded all BP measurements. Brachial and central BP and AIx were assessed by non-invasive applanation tonometry using the SphygmoCor (AtCor Medical, Australia). The SphygmoCor quantifies radial arterial blood pressure and waveform reflections using a validated generalised transfer function to accurately measure central BP and AIx.[683]

Subjects' cardiorespiratory fitness, determined as peak oxygen consumption ($\dot{V}O_{2peak}$), was assessed through a maximal graded treadmill or cycle ergometer test via pulmonary analysis. While control subjects completed a maximal treadmill test, the endurance-trained cyclist completed a cycle ergometer test. Triathletes obtain a comparable $\dot{V}O_{2peak}$ value regardless of

exercise mode[301] and as such, triathletes from the present study completed either a cycle or treadmill test. Before maximal exercise testing subjects were fitted with a two-way breathing valve (Hans Rudolph) and expired air was collected into an online metabolic system (Moxus, Modular) for O_2 and CO_2 analysis. The metabolic system was calibrated prior to each test using ambient air and gas of known composition. The treadmill commenced at 10 $km \cdot h^{-1}$ and was progressively increased by 1 $km \cdot h^{-1}$ every two minutes until volitional exhaustion. Cycle ergometer $\dot{V}O_{2max}$ tests commenced at 100 W and the load was increased by 30 $W \cdot min^{-1}$ every two minutes until pedalling cadence dropped below 50 RPM for 10 seconds or until volitional exhaustion. Subjects were asked to maintain 90–100 RPM throughout cycle ergometer-assessed exercise tests. Individual $\dot{V}O_{2peak}$ was determined as the highest O_2 value averaged over 60 seconds.

Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics for Windows (Version 21, IBM Corp, NY). A 10% absolute increase in central AIx is associated with a 32% and 38% increased risk of future cardiovascular events and all-cause mortality, respectively.[673] Our *priori* power analysis revealed that in order to detect a 10% difference (Cohen's $d = 0.8$) in AIx between athletes and controls, we required 42 subjects in each group (n = 84) to achieve >90% power (two-sided alpha = 0.05) (G*Power 3.1.5). Data were tested for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Two-tailed independent samples *t*-tests or Mann-Whitney U-tests were used to examine physical, hemodynamic and fitness differences between athletes and controls. To control for covariates, an ANCOVA was used to establish differences between athletes and control BP and AIx. Bonferroni-corrected Pearson's and Spearman's Correlation *p*-values were used to determine statistically significant associations between brachial BP, physical activity, $\dot{V}O_{2peak}$, central SBP and AIx. Stepwise linear

regression was performed to identify predictors of AIx. Statistical significance was set at $p < 0.05$.

RESULTS

The physical characteristics of athletes and controls are outlined in Table 1. Relative to the controls, athletes had a lower body weight, BMI, brachial systolic and diastolic BP and mean arterial pressure (MAP), central systolic BP (SBP) and resting heart rate, were less physically inactive (sitting) and had higher cardiorespiratory fitness, expended more energy and were more physically active (all $p < 0.05$, Table 1). Brachial systolic and diastolic BP (DBP) and central SBP remained significantly different after adjusting for age ($p = 0.02$, $p = 0.002$ and $p = 0.004$, respectively). Considering AIx is sensitive to age, resting heart rate and height[670] and the linear relationships between age, height and AIx (Figure 1), we compared AIx between athletes and controls after adjustment for these variables. Age, height and resting heart rate-adjusted AIx was lower in athletes compared to controls (athletes vs controls mean [%] ± SE [95% CI]: -3.7 ± 2.04 [-7.79 – 0.31] vs 6.9 ± 1.87 [3.15 – 10.62], $p = 0.001$, Figure 2). This difference remained statistically significant after further adjustment for BMI, brachial SBP and DBP, and central SBP (-0.56 ± 1.29 [-3.12 – 2.01] vs 4.09 ± 1.17 [1.75 – 6.42], $p = 0.03$). The lower AIx observed in athletes compared to controls were however ameliorated in the fully adjusted model when accounting for $\dot{V}O_{2peak}$ (0.73 ± 1.45 [-2.16 – 3.62] vs 3.19 ± 1.29 [0.62 – 5.76], $p = 0.31$).

Table 2 outlines results from Pearson's or Spearman's Correlations that were performed to determine associations between physical activity and hemodynamic parameters in athletes and controls. $\dot{V}O_{2peak}$ was inversely correlated to brachial DBP and AIx (r = -0.27, $p = 0.01$ and r = -0.45, $p = 2.0 \times 10^{-5}$, respectively, Table 2). While weekly estimated energy

expenditure was positively associated with brachial pulse pressure (r = 0.34, p = 0.005), weekly METs were negatively correlated to brachial diastolic BP (r = -0.31, p = 0.01, Table 2). No statistically significant correlations were observed between physical activity parameters, BP measures or AIx (Table 2).

We then performed a stepwise regression to explore what blood pressures (brachial SBP/DBP and central SBP) physical (age, height, weight and BMI) and fitness parameters (resting HR and $\dot{V}O_{2peak}$) predicted AIx. Notably, $\dot{V}O_{2peak}$, brachial SBP and DBP, and central SBP emerged as independent predictor of AIx, and together explained 81% of the variance (Table 3).

When accounting for age, we found men had higher cardiorespiratory fitness compared to women (Men vs Women mean $\dot{V}O_{2peak}$: 42.7 vs 51.7 $ml \cdot kg^{-1} \cdot min^{-1}$, p = 0.0003) and larger studies have reported a similar gender difference (45.4 vs 37.0 $ml \cdot kg^{-1} \cdot min^{-1}$, n = 3678),[684] we conducted the same analysis with men and women separate. Relative to those men with low cardiorespiratory fitness (mean AIx [%] ± SE [95% CI]: 9.65 ± 3.14 [3.39 – 15.92], n = 17), those with moderate and high fitness had lower AIx (mean AIx [%] ± SE [95% CI]: -1.16 ± 2.02 [-5.19 – 2.87], n = 32, p = 0.004 and (-4.00 ± 2.62 [-9.23 – 1.23], n = 25, p = 0.004); yet no difference were found between moderately and highly fit men (p = 0.41, Figure 3A). When we performed the same analysis in women, we observed no statistically significant differences in AIx between subjects in cardiorespiratory fitness tertiles (p = 0.2, Figure 3B); a result probably owing to a much smaller sample size (n = 20).

DISCUSSION

The aim of our study was to explore differences in BP (brachial and central) and AIx between highly endurance-trained athletes and recreationally active controls, and to determine whether differences were influenced by $\dot{V}O_{2peak}$ or physical activity parameters. A novel finding of

this study is that AIx are lower in highly endurance athletes compared to recreationally active individuals and that the difference in AIx was partially mediated by $\dot{V}O_{2peak}$. We also found linear correlations between BP indices and AIx, physical activity parameters and $\dot{V}O_{2peak}$. Interestingly, $\dot{V}O_{2peak}$ emerged as independent predictor of AIx and when analysed in conjunction with brachial SBP and DBP, and central SBP, accounted for 81% of the variance. Finally, our study is the first to demonstrate that men with moderate and high $\dot{V}O_{2peak}$ defined as 45–55 and 55+ $ml \cdot kg^{-1} \cdot min^{-1}$, respectively, exhibited lower AIx compared to their less-fit (<45 $ml \cdot kg^{-1} \cdot min^{-1}$) peers.

The reduction in cardiovascular disease and mortality risk through regular aerobic exercise training is undoubtable,[75,685] yet whether high amounts of endurance exercise further benefits cardiovascular health is unclear. Indeed, chronic endurance exercise training for triathlons, ultra/marathons and endurance cycling events could cause an elevated risk for cardiovascular complications, in particular, stiffening of the heart and large arteries.[677] Elevated central BP (a correlate of pulse wave velocity) exerts adverse load on the heart and aorta during systole,[670] which could inevitably contribute to the downstream sequalea of cardiac strain. We found that individuals regularly performing endurance exercise training exhibit significantly lower AIx. Furthermore, the endurance athletes had significantly lower brachial and central BP (SBP, DBP and MAP) compared to recreationally active subjects. Considering the predictive value of central BP and AIx on future cardiovascular disease, outcomes[667,671,672] and mortality risk,[666,673] the lower central hemodynamic profile observed in the athletes from our study indicates these athletes may have ameliorated risk for future adverse cardiovascular events and mortality.

Whilst our data corroborates previous studies[678,686] on AIx in endurance athletes, it refutes others.[674,679,680,682] Current data on central BP between endurance athletes and controls are

equally inconclusive.[674,678,680-682,687] These inconsistencies could partly be explained by subject ages, exercise habits and training histories, $\dot{V}O_{2peak}$ differences and also by methodological consideration, including timing of BP parameter measurements (i.e. day before competition). Central AIx is a more sensitive marker of arterial stiffening in individuals younger than 50 years old,[688] which could also account for some discrepancies throughout the current literature.

Intriguingly, the significantly lower AIx observed in the athletes compared to controls was ameliorated after $\dot{V}O_{2peak}$ adjustment, indicating the difference was dependent on $\dot{V}O_{2peak}$. Interestingly, data from a recent meta-analysis of randomised controlled trials revealed a dose and intensity-dependent relationship in the aerobic exercise-induced reduction in wave reflections.[689] We did not find any correlations between energy expenditure parameters (METs/Kj/training/sitting) and AIx. We did, however, find linear correlations between objectively assessed $\dot{V}O_{2peak}$ and AIx. Whereas $\dot{V}O_{2peak}$ was independently associated with AIx previously, it was not analysed in context with athlete and control status.[686] Thus, we have verified that $\dot{V}O_{2peak}$ is an independent predictor of AIx, and partly explains the differences in AIx between endurance athletes and controls.

We are the first to show individuals with low $\dot{V}O_{2peak}$ have higher AIx compared to those with moderate or high $\dot{V}O_{2peak}$. This novel finding supports the concept that $\dot{V}O_{2peak}$ may be an unmeasured confounding factor in previous cross-sectional studies including endurance-trained individuals.[674,679,680] This finding suggests that healthy individuals may not require an exceptionally high $\dot{V}O_{2peak}$ to lower AIx; rather a more achievable (45–55 $ml \cdot kg^{-1} \cdot min^{-1}$) cardiorespiratory fitness may be enough. The risk of cardiovascular disease development[690,691] and cardiovascular disease-related mortality[692,693] is markedly attenuated by possessing higher $\dot{V}O_{2peak}$.

A higher $\dot{V}O_{2peak}$, reflects the cardio-vascular and muscle skeletal adaptations associated with regular exercise training. Acute[694-696] and long-term[565,697] exercise training also increases circulating endothelial progenitor cells (EPCs) that have a known function in maintaining the integrity of the inner arterial wall. Elevated laminar shear stress, the subsequent release of endothelial nitric oxide (eNO) by nitric oxide synthase (eNOS) and increased circulating EPCs caused by repeated, prolonged bouts of endurance exercise,[233] could account for the lowered BP and AIx observed in the endurance athletes. Alternatively, chronic endurance exercise training reduces circulating inflammation and oxidative stress; [202,698] both of which contribute to arterial stiffening.[699]

The relatively large sample size and adequate statistical power are strengths of our study. The athletes and controls had their BP and AIx measured in a rested condition and not before an endurance event. Furthermore, we completed a comprehensive analysis on AIx after adjusting for confounding variables – height, resting heart rate and brachial and central BP. We do, however, acknowledge that like every study, ours has some limitations. First, our study included correlations and cross-sectional analyses, and as such causality cannot be assumed. It is, however, likely that the endurance athletes lowered BP and AIx are due to cardiorespiratory fitness and health benefits adaptations from long-term involvement in exercise training and data from randomised controlled trials supports this claim.[689] We did not quantify dietary habits, insulin resistance or control for the effects of the menstrual cycle on central BP and AIx in our subjects. Further, we were underpowered to detect a difference between cardiorespiratory fitness tertiles and AIx in women. Therefore, this deserves attention in future investigations. Future research is also warranted to elucidate the mechanisms responsible for the impact of cardiorespiratory fitness on BP and AIx and may include eNO availability, decreased sympathetic tone, and molecular mechanisms such as microRNA molecules, telomere regulation or epigenetics.

In conclusion, we have shown for the first time that the lower aortic AIx in endurance athletes compared to controls is partly mediated by $\dot{V}O_{2peak}$. We also demonstrate that moderate cardiorespiratory fitness is associated with decreased aortic AIx, an affect not augmented by possessing a higher $\dot{V}O_{2peak}$. Together, these data suggest that regular endurance exercise training is associated with lower central BP and AIx, but also emphasises that a cardiorespiratory fitness of 45–55 $ml \cdot kg^{-1} \cdot min^{-1}$ is an achievable target associated with low AIx. Given the relationship between AIx, cardiovascular disease and ageing, health professionals should advocate aerobic exercise aimed at improving $\dot{V}O_{2peak}$.

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CONFLICT OF INTEREST

We have no conflict of interests to declare.

SUMMARY TABLE

What is known about this topic

- Aerobic exercise training improves cardiovascular health though the ideal dose is currently unknown.
- Data on aortic AIx in endurance athletes have been equivocal.

What this study adds

- This study shows correlations between exercise training, blood pressure parameters and AIx.

- After controlling for confounding variables, this study shows that endurance athletes have significantly lower AIx and that this effect is likely explained by cardiorespiratory fitness.
- Relative to unfit subjects, lower AIx are observed in those with moderate and high cardiorespiratory fitness to a comparable level.

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FIGURE LEGENDS

Figure 1. Older and shorter subjects exhibit higher AIx. Pearson's correlation coefficients of AIx with age (A) and height (B).

Figure 2. Augmentation index difference between athletes and controls. Data are expressed as mean ± standard error from ANCOVA adjusted for age, height and resting heart rate. Athletes have lower AIx compared to controls (athletes vs controls mean [%] ± SE [95% CI]: -3.7 ± 2.04 [-7.79 – 0.31] vs 6.9 ± 1.87 [3.15 – 10.62], $p = 0.001$).

Legend: AIx, augmentation index; ** $p < 0.01$ vs athletes.

Figure 3. Impact of low, moderate and high cardiorespiratory fitness on AIx in men and women. Data are expressed as mean ± standard error from ANCOVA adjusted for age, height and resting heart rate. A) Compared to men with low (<45 $ml \cdot kg \cdot min^{-1}$, n = 17) cardiorespiratory fitness, those with moderate (45.1–55 $ml \cdot kg^{-1} \cdot min^{-1}$, n = 32) and high (55.1+ $ml \cdot kg^{-1} \cdot min^{-1}$, n = 25) fitness had lower AIx (low vs moderate vs high; mean [%] ± SE [95% CI]: 9.65 ± 3.14 [3.39 – 15.92] vs -1.16 ± 2.02 [-5.19 – 2.87] vs (-4.00 ± 2.62 [-9.23 – 1.23], $p = 0.007$). B) Compared to women with low (<35 $ml \cdot kg \cdot min^{-1}$, n = 7) cardiorespiratory fitness, those with moderate (35.1–45 $ml \cdot kg^{-1} \cdot min^{-1}$, n = 5) and high (45.1+ $ml \cdot kg^{-1} \cdot min^{-1}$, n = 8) fitness had lower AIx (low vs moderate vs high; mean [%] ± SE [95% CI]: 14.95 ± 5.87 [2.35 – 27.55] vs 13.01 ± 6.07 [0.003 – 26.03] vs -2.09 ± 5.78 [-14.48 –10.3], $p = 0.2$).

Legend: AIx, augmentation index; ** $p < 0.01$ vs < 45 $ml \cdot kg \cdot min^{-1}$.